Transcriptomic Response of Porcine PBMCs to Experimental Tetanus Vaccination: Comparison of Divergent Phenotypes for Lean Growth and Antibody Titers

Dissertation

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1 Introduction

1.1 Breeding of livestock animals

The domestication of farm animals began more than ten thousand years ago and, considering genetic selection by current breeding programs, is still going on today. As defined by the United Nations Convention on Biological Diversity a domesticated animal is a "species in which the evolutionary process has been influenced by humans to meet their needs" (Article 2, Use of Terms). Selective breeding of livestock such as cattle, pig and poultry is focussed on phenotypic traits of interests for human consumption such as body and carcase composition, weight gain, milk or egg performance and behaviour; whereas other biological traits are not regarded for various reasons. Modern animal breeding is based on selection of animals with known genetic values responsible for the desired phenotypic traits. These genetic values are referred as to estimated breeding value, EBV and are determined by best linear unbiased prediction (BLUP) or other statistical methods (Kräußlich 1997). The resulting breeding programs caused gradually accumulated genetic changes, the most of them are associated with desired phenotypic alterations (Merks 2000). However, also several negative side effects became known concerning behaviour, stress resistance, physiological parameters and increased incidences of infectious or autoimmune diseases due to intensive selection for productive traits (Rauw *et al.* 1998, Prunier *et al.* 2000).

Considering their genetic diversity, domesticated species often went through a domestication bottleneck (Chen *et al.* 2007, Wiener and Wilkinson 2011): a process of rapid decrease of the effective population size followed by a reduction of genetic variation. However, although additionally artificial selection and inbreeding will reduce genetic diversity, most domesticated animals still show a considerably high genetic variation (Wiener and Wilkinson 2011).

The main focus of this thesis is on the domestic pig, *Sus scrofa domesticus*, that was domesticated from the wild boar, *S. scrofa*, about 9.000 years ago (Bökönyi 1974, Giuffra *et al.* 2000). Unlike most other domesticated animals pig domestication occurred at different times independently in several regions of Europe and Asia (Kijas *et al.* 2001, Larson *et al.* 2005). The porcine domestication process resulted in considerable phenotypic changes of behaviour, coat color, body composition and, a striking morphological difference to the wild boar, back elongation due to an increased number of vertebrae (King and Roberts 1960).

Domestic pigs are represented by a large number of breeds and their considerable genetic and phenotypic diversity (Chen *et al.* 07 and Li *et al.* 2014). One major reason for this is due to the introduction of Asian pig breeds into Europe during the 18th century followed by introgressive hybridization (Giuffra *et al.* 2000, Kijas *et al.* 2001).

In the past of pig farming until the middle of the 20th century the demand was for fatness but since several decades pigs are selected strongly for lean growth, i.e. high muscle and low fat content (Fowler *et al.* 1976, Schinckel and de Lange 1996, Rubin *et al.* 2012). During this period the first breeding programs and companies have emerged (Merks 2000). By use of cross breeding and, since the last two decades, by application of molecular genetics and statistics further production traits were gradually improved such as reduced backfat thickness, daily weight gain, feed efficiency and litter size.

Nowadays, in addition to these strictly economic traits, so-called societally important traits (Kanis *et al.* 2005) have become more important and are proposed to be included into breeding objectives (Merks 2000, Merks *et al.* 2012, Hayes *et al.* 2013). These traits are due to public concerns and contain economic and noneconomic values: health, vitality and welfare, ecological effects and carbon footprint of pork production as well as pork quality and healthiness.

Since infectious diseases cause mortality, decreased vitality and welfare, reduced production efficiency but increased costs the issue of general disease resistance is important for both, economic production and health and welfare and thus has become of major interest in pig farming and breeding. Therefore, genetic resistance to infectious diseases has been strongly recommended to be included into breeding (Wilkie and Mallard 1999, Stear *et al.* 2001, Lewis *et al.* 2007, Reiner 2009). However, there is still a lack of understanding the genetic relationship between immune traits that are associated with disease resistance and production or economic performance traits such as weight gain or lean growth.

1.1.1 Genetic relationship between immune and production traits

Animal health and welfare on the one hand as well as product quality and economic efficiency on the other hand are interdependent and both are reliant on the prevention of clinical and subclinical pathogen infection. Since both intensive and organic pig production systems are faced with a number of different pathogens and public concern on the use of antibiotics and other drugs has increased there is a demand for new breeding approaches that sustain the genetic resistance to infectious diseases (Reiner 2009, Merks *et al.* 2012). Selection for high immune response seems feasible since considerable genetic variation of porcine immune

response has been shown (Edfors-Lilja *et al.* 1994, Henryon *et al.* 2001, de Groot *et al.* 2005, Flori *et al.* 2011).

However, the direct genetic relationship between immune responsiveness and performance traits is not well understood and little is known about possible side-effects due to intensive selection of high immune response genotypes. There is an extensive research record on how such factors as pathogen infection, poor hygienic environments and physical or psychological stressors adversely affect metabolism and thus lead to lower animal performance (Colditz 2002). Immune responses are metabolically expensive and are expected to require reallocation of metabolic resources (Rauw 2009, Rauw 2012) possibly at the expense of growth or gain performance. However, these observations of metabolic impairments due to an activated immune system should be differentiated from genetically determined differences in immune responsiveness and performance traits and the genetically controlled balance between them.

In the early 1970s Biozzi and coworkers established a mouse model with divergent genetic lines of high (Ab/H) and low (Ab/L) antibody response to sheep erythrocytes (Biozzi *et al.* 1970, Biozzi *et al.* 1972). Ab/H and Ab/L were shown to possess also high and low humoral defense to other unrelated antigens than sheep erythrocytes (Biozzi *et al.* 1975). Furthermore, Ab/H mice better resist extracellular infections but were more susceptible to intracellular infections by *Salmonella typhimurium* due to decreased macrophage function (Biozzi *et al.* 1979, Dockrell *et al.* 1985). Hence, in Biozzi mice genetic selection towards high humoral immune response was accompanied by the side-effect of weaker cellular immune defense. An overview of published research on the relationship between immune traits and livestock performance is given by table 1. In chickens and turkeys, it is well documented that the selection for high growth rates have led to an impaired immune competence and thus higher incidences of infectious diseases (Bayyari *et al.* 1997, Rauw *et al.* 1998, Swaggerty *et al.* 2009).

One of the first detailed studies on this issue in swine has reported a negative correlation between weight gain and antibody titers due to vaccination against bacterial and viral pathogens (*Bordetella bronchiseptica* and pseudorabies virus) in crossbred pigs (Meeker *et al.* 1987). Later Mallard, Wilkie and colleagues generated genetic lines of high and low innate and adaptive immune responsiveness by selective breeding of Yorkshire pigs (Mallard *et al.* 1992). Genetic lines of high humoral and cellular immune responses were associated with enhanced weight gain (Mallard *et al.* 1998, Wilkie *et al.* 1998). However, these high immune response animals were more prone to suffer from arthritis (Magnussen *et al.* 1998).

More recently, Clapperton and colleagues found that in Large White pigs selected for high or low lean growth under restricted feeding (Cameron 1994), high lean growth animals had higher numbers of of the acute phase protein AGP and several lymphocyte and monocyte subsets, although under *ad libitum* feeding no associations were observable (Clapperton *et al.* 2005,

Clapperton *et al.* 2006). Further research on commercial populations demonstrated that many leukocyte subsets are heritable and negatively correlate, phenotypically and genetically, with daily gain performance.

Likewise, negative correlations were seen between average daily weight gain and quantities of several lymphocyte subsets, although proportions of SLA-DQ-positive cells were positively correlated with carcass weight and feed conversion (Galina-Pantoja *et al.* 2006).

1.1.2 Overview of literature

Table 1. Overview of important literature on reciprocal effects between animal performance and/or immune responsiveness

Time period	Animal experiment	Observations	Literature
1970s and 1980s	Biozzi mice: Selective breeding of mice yielded divergent genetic lines of high and low AB titers against sheep erythrocytes	High responders also generate high AB titers in response to other unrelated antigens, high AB responders were better protected against extracellular infections. However, high responders showed impaired macrophage function and therefore point to an inverse relationship between humoral and cellular immunocompetence.	1
1990s	Commercial breeding of chickens and turkeys	Selection for growth production performance led to impaired immunocompetence and decreased resistance to infectious diseases. Moreover, selection for higher immune responsiveness was accompanied with reduction of body weight.	2
1980s	Crossbred pigs: Vaccination against *Bordetella bronchiseptica* and Pseudorabies.	Negative correlation between antibody titers and weight gain. No association between antibody titers and backfat thickness.	3

Table 1. Continued

Time period	Animal experiment	Observations	Literature
1990s	Yorkshire pigs: bred to derive high (HIR), low (LIR) and control lines of antibody and cell-mediated immune response.	HIR animals showed higher antibody responses to various antigens. Selection for HIR was associated with enhanced weight gain, but HIR was also correlated with the incidence of severe arthritis.	4
Since 2000s	Large White pigs: selection of divergent lean growth under restricted and *ad libitum* feeding and divergent food intake yielding six genetic lines: high and low lean growth under restricted feeding, high and low lean growth under *ad libitum* feeding and high and low food intake.	Animals selected for high lean growth under restricted feeding have shown (1) higher levels of the acute phase protein alpha1-acid glycoprotein (AGP) (2) higher levels of white blood cell count with higher numbers of lymphocytes, in particular CD8a+ and CD11R1+ (NK) cells No such association was found for high lean growth selected under *ad libitum* feeding or divergent food intake.	5
	Large White pigs from commercial farms	Quantities and proportions of numerous immune traits such as (1) total white blood cell count (2) CD4+, CD8a+, CD11R1+ (NK), gamma delta T cell receptor+ cells (3) B cells and Monocytes (4) several acute phase proteins are heritable, and were genetically and phenotypically negatively correlated with daily gain performance.	6

Table 1. Continued

Time period	Animal experiment	Observations	Literature
Since 2000s	Crossbred pigs from a commercial farm	Proportions of several T lymphocyte subsets, i.e. CD16+, CD2+/CD16+ (NK), and CD8+ were negatively associated with average daily gain performance. However, numbers of SLA-DQ+ cells were positively correlated with higher carcass weight and feed conversion.	7

Legend to references:
1. Biozzi *et al.* 1970, 1972, 1975, 1979, Dockrell *et al.* 1985
2. Bayyari *et al.* 1997, Rauw *et al.* 1998, Swaggerty *et al.* 2009
3. Meeker *et al.* 1987
4. Mallard *et al.* 1992, 1998, Wilkie *et al.* 1998, Magnusson *et al.* 1998
5. Cameron 1994, Clapperton *et al.* 2005, 2006
6. Clapperton *et al.* 2008, 2009
7. Galina-Pantoja *et al.* 2006

1.1.3 The domestic pig as biomedical model animal

As one of the most productive protein sources for human nutrition the pig is an important agriculture animal. Moreover, the interest on the use of swine as biomedical model animal has been devoted early (Swindle 1992, Swindle *et al.* 1994) and was reinforced during emergence of functional genomics (Lunney 2007, Bendixen *et al.* 2010).

Although the well known standard models in biology, e.g. fruitfly, zebrafish and mouse, have the advantages of being genetically well characterized and annotated at the ‘omics scale as well as being easily kept and available in high numbers in laboratory facilities in some cases they do not sufficiently reflect human biology (Bendixen *et al.* 2010). There are several biological properties and similarities of pig to human that make it an "excellent biomedical model" (Lunney 2007). Striking advantages of porcine models are their highly comparative physiology, genetics, size and anatomy to human. Medical and bioscientific branches that refer to porcine models are remarkably diverse including cardiovascular and pulmonary physiology, digestive physiology and nutrition, reproduction and urogenital system, musculoskeletal

system and biomechanics, transplantation and tissue engineering, brain and nervous system as well as immunology and infectious diseases.
The pig genome is fully sequenced, the current draft genome assembly (10.2) is available at the Ensembl database (http://www.ensembl.org/Sus_scrofa). At the genetic and genomic scales high homologies of DNA sequence and chromosome structure and hence similarities of transcriptome and proteome are the reason why pig are utilized as model for, amongst others, melanoma (Zhi-Quiang *et al.* 2007) and infectious diseases (Elahi *et al.* 2005, Dvorak *et al.* 2006). The completion of the porcine genome is a crucial step forward for developing human disease models by usage of comparative genomics (Walters *et al.* 2012).

1.2 The immune response to Tetanus Toxoid

This thesis and it's studies are based on the application of tetanus toxoid (TT) vaccine as experimental antigen to induce a comprehensive cellular and humoral immune response. TT is derived from the corresponding toxin tetanospasmin or tetanus toxin which is produced by the bacterium *Clostridium tetani*. *C. tetani* is a soil-living, gram-positive, anaerobe and spore-forming organism. Infection occurs through soil-contaminated skin lessions in particular deep wound punctures (Madigan and Martinko 2005). Germination of spores and bacterial proliferation is initiated in such anoxic areals while *C. tetani* itself is noninvasive. Following the bacterial proliferation several toxins are produced of which tetanospasmin is causative for tetanus disease. Tetanus toxin belongs to the A-B class of bacterial exotoxins. The toxin interacts with neurons and spreads into the central nervous system where it causes a rigid spastic paralysis of skeletal muscles (Mellanby and Green 1981, Pelizzarri *et al.* 1999). Tetanus can be successfully prevented by active vaccination with tetanus toxoid which is derived from the toxin by treatment with formalin.
Although all mammal species are susceptible to the disease prevention by vaccination is of more importance in certain livestock species such as horses and ruminants than in others. In swine tetanus is not of major importance and therefore pigs are not vaccinated as part of standard farming practice. Since in conventional pig farming TT represents a non-ubiquitous antigen in particular weaning piglets are considered to be antigen-naïve (Ponsuksili *et al.* 2008).
The immune response to tetanus toxoid involves antigen uptake and processing by antigen presenting cells via both major histocompatibility complex (MHC) complexes I and II followed by interaction with CD4 as well as CD8 T-cell clones (Kerblat *et al.* 2000). Antigen presenting

cells process antigens to peptide fragments and present them to T-cells in two different ways (Janeway et al. 2002, Madigan and Martinko 2005): MHC class I molecules display antigenic peptides derived from intracellular cytosolic proteins which originate from host body cells or intracellular pathogens. The antigen loaded MHC I complex interacts exclusively with the T-cell receptor (TCR) of CD8-positive T-cells, these CD8+ cells or cytotoxic T lymphocytes (CTL) induce apoptosis of the antigen presenting cell (Kaufmann 1988).

The other pathway of antigen presentation involves MHC class II complexes which interact exclusively with T-cells positive for the CD4-coreceptor. MHC II antigen processing is restricted to professional antigen-presenting cells and is directed to endocytosed extracellular antigenic peptides. The binding of MHC II to the TCR of naïve T cells (TH0) leads to differentiation into two major types: type I and type II (Mosmann *et al.* 1986). Referred also as TH1 (T-Helper type 1) and TH2 these pathways differ in the spectrum of cytokines secreted by activated T helper cells and the resulting activation of defense mechanisms against intracellular or extracellular pathogens, respectively (Mosmann and Coffman 1989). TH1 cells secrete IL-2, IFN-γ and TNF-α/β. The main effector cytokine IFN-γ is directed to macrophage activation and TH2 inhibition. TH2 helper cells are triggered by IL-4 and produce IL-4, IL-5, IL-6 and IL-13. The key cytokine IL-4 stimulates B cell proliferation and inhibits the TH1 pathway.

The TH1/TH2 mechanism was discovered and is best characterized in mice (Mosmann *et al.* 1986, Mosmann and Coffman 1989). Although it has been shown that the major effector cytokines have the same or similar function in swine it is not yet fully clarified to what extent the paradigm applies in swine. Murthaugh *et al.* (2009) reported evidence that porcine IL-4 does not stimulate B cell proliferation and thus point to a different function of IL-4 than in mice or humans. The TH1/TH2 dichotomy requires a balance of cytokine production to provide protection against both intra- and extracellular pathogens, however there exists a appreciable individual variation in swine of either predominantly TH1 or TH2 or equilized cytokine production (de Groot *et al.* 2005).

Summarising the above, TT induces a combined humoral and cellular immune response, involving CD8+ cells, TH1 and TH2 cells (ElGhazali *et al.* 1993, Xu-Amano *et al.* 1993, Regnström *et al.* 2002, Regnström *et al.* 2003). Hence, TT is a very suitable experimental antigen (Kerblat *et al.* 2000, Nookala *et al.* 2004), particularly in swine (Ponsuksili *et al.* 2008) because it is non-ubiquitous and weaning piglets can be considered as antigen-naïve. Therefore, an experimental vaccination with the TT antigen will most likely be the first immune challenge with TT and thus will not be biased by adaptive responses due to past or present infections with *C. tetani*.

1.3 PBMC transcriptome analysis

Differential gene expression analysis is widely applied for characterization of organismal responses to environmental stimuli. Increase or decrease of gene product frequencies, i.e mRNA abundances, are due to events of gene regulation and/or proliferation of certain cell types expressing the respective genes. In the context of the immune response such analyses can be directed to lymphoid tissue (Wang *et al.* 2007, Wang *et al.* 2008, Malhotra *et al.* 2012) or, and more frequently, to peripheral blood (Ponsuksili *et al.* 2008, Gao *et al.* 2010, Flori *et al.* 2011). As major effector of the immune system peripheral blood leukocytes are studied in cell culture via mitogenic stimulation (Gao *et al.* 2010, Wilkinson *et al.* 2012) or in vivo by experimental pathogen infection (Ponsuksili *et al.* 2008).

Peripheral blood mononuclear cells (PBMCs) are obtained by density gradient centrifugation (English and Andersen 1974) that separates a whole blood sample into plasma, mononuclear cells (thrombocytes, lymphocytes, monocytes, dendritic cells and a part of basophils) and polymorphonuclear (neutrophils, eosinophils and erythrocytes) cells (Janeway *et al.* 2002; Figure 1). Thus, the PBMC fraction contains all leukocytes that have a round nucleus including lymphocytes, monocytes and dendritc cells. The frequencies of PBMC subsets vary between mammal species (Kraft and Dürr 1999) but the overall pattern is the same. Lympocytes are present at approximately 70 - 90 % (in human) followed by monocytes (5 - 30 %) and dendritic cells (< 2 %) (McLaren *et al.* 2004, Miyahira 2012). The lymphocyte fraction in turn is composed of T cells (CD4-positive ~ 50% in human, ~ 30 % in pig; CD8-positive ~ 25 % in human, ~ 10% in pig), B cells (~ 15 % in human, ~ 28 % in pig) and NK (natural killer) cells (~10% in human) (Huang *et al.* 1995, Porrata *et al.* 2001, McLaren *et al.* 2004, Pasternak *et al.* 2014). An implication of this lymphocyte frequency pattern for micorarray analysis is discussed in section 3.1.2. Due to their easy isolation and the easy availability of blood PBMC targeted approaches are widely used in immunological research and clinical diagnostics. In the past studies of transcriptional response of PBMCs to immune challenges were limited to the analysis of single candidate genes. In studies of porcine immune responses the interest was initially focussed on cytokine genes (Yancy *et al.* 2001, Verfaillie *et al.* 2001, Suradhat and Thanawongnuwech 2003, Suradhat *et al.* 2003). The intense study of single genes enables a detailed and accurate characterization of gene expression. By analyses of multiple sampling intervals time course studies can determine the onset and kinetics of immune responses (Choi *et al.* 2002). However, by analysis of few candidates any alteration of other genes that may be co-involved will remain unrecognized.

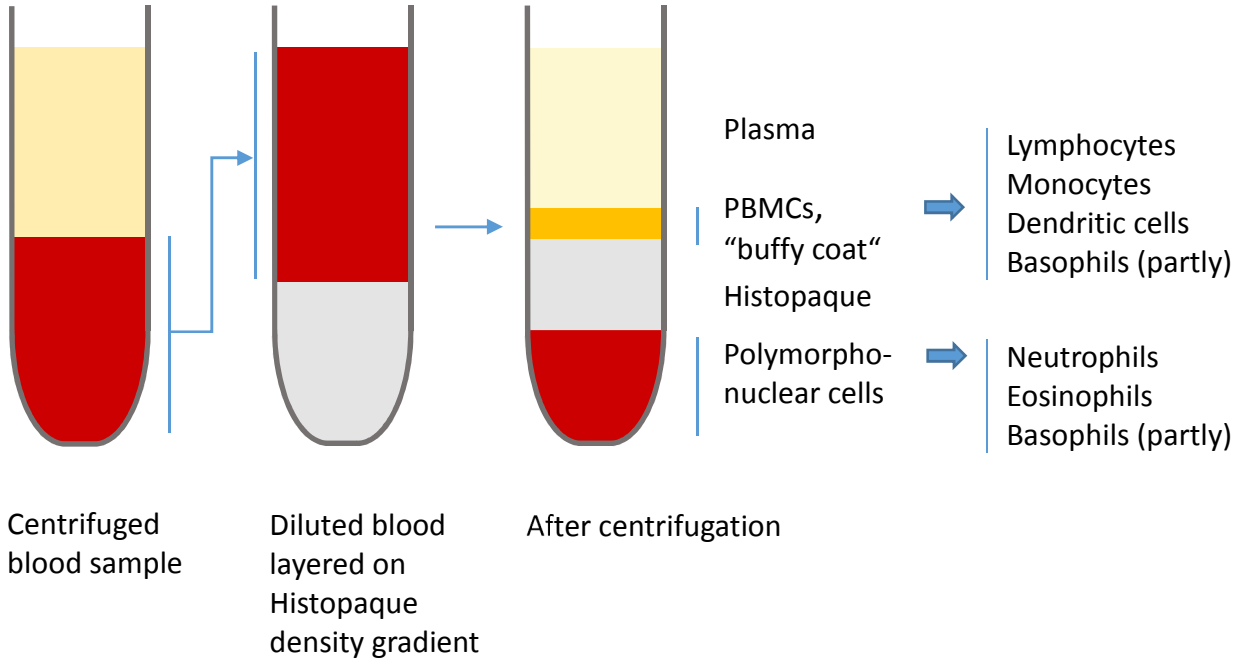

Figure 1. Isolation of peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation. Diluted blood is layered on a Histopaque density gradient (1077 g/mL) and centrifuged. The sample tube will contain a plasma layer on top, the "buffy coat" layer representing the mononuclear cells and a bottom layer composed of the denser polymorphonuclear cells.

Since the advent of DNA microarrays (Schena *et al.* 1995) the transcriptomics approach complements and extends the analysis of functional genes after experimental immune stimulation (Ponsuksili *et al.* 2008) or pathogen infection (Tuggle *et al.* 2007, Tomas *et al.* 2010, Huang *et al.* 2011). Modern microarrays enable the simultaneous interrogation of high gene numbers, for instance by custom-made arrrays targeting immune-specific genes (Gao *et al.* 2010) or reflecting the whole genome with ten thousands of genes (Freeman *et al.* 2012). However, also some adverse side effects of microarray techniques have to be taken into account which will be discussed in section 3.1. With Next Generation Sequencing (NGS) a recent advancement in transcriptomic research has emerged (Mutz *et al.* 2013).

Transcriptomics is part of functional genomics (Hieter and Boguski 1997) which means the integration of genomic, transcriptomic and proteomic data in order to gain understanding of gene function and interaction. In this context, transcriptome profiling of PBMCs enables the characterization of transcriptional shifts due to immune challenges. Alterations of transcript abundances can be due to either gene regulation events or changes of proportions of cell types which express the respective gene. After bioinformatical analysis of these affected genes the

identification of biological functions, i.e. signaling pathways, allows the characterization of immune responses and further changes of cellular metabolism and signaling.

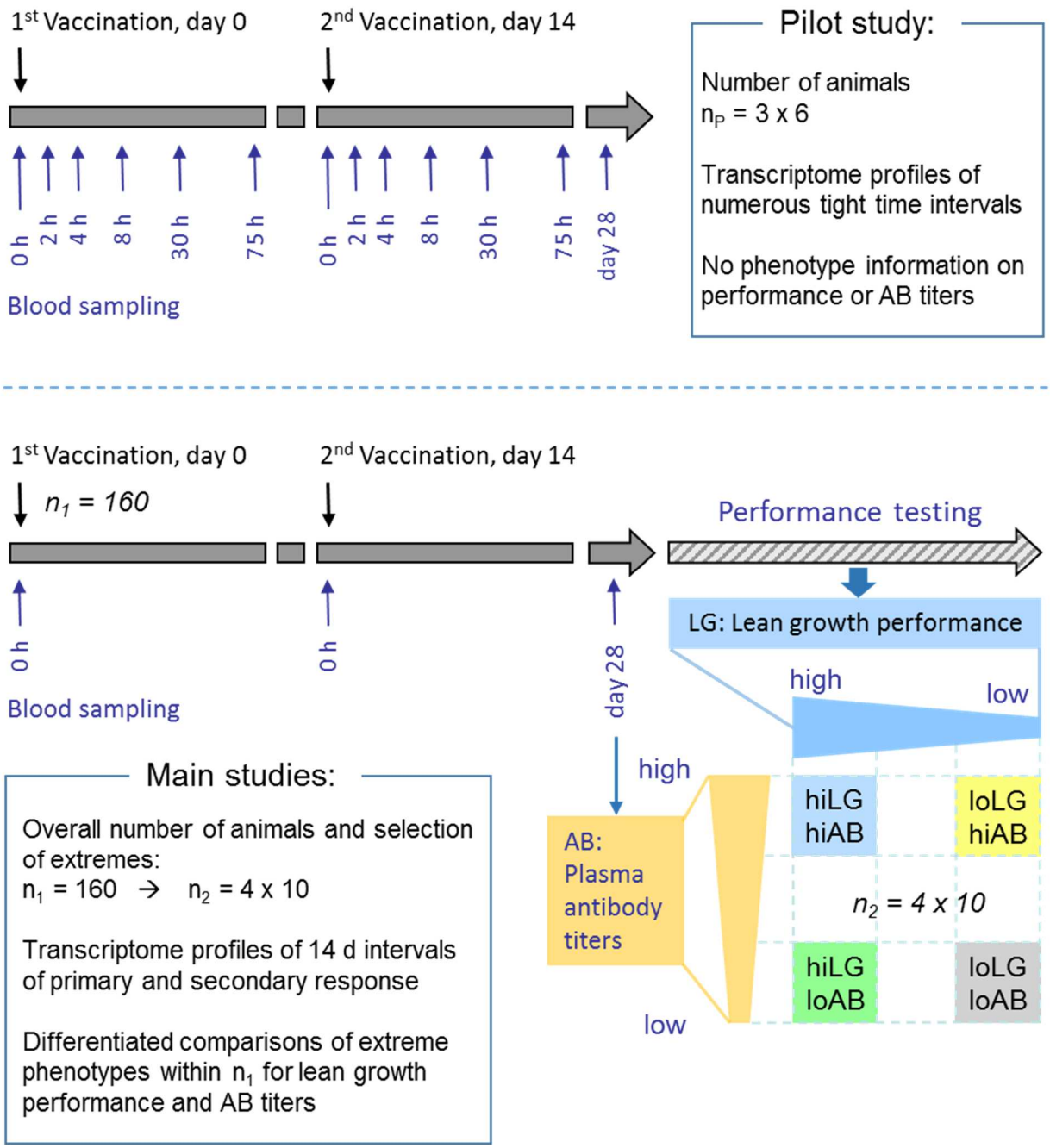

Figure 2. Overall experimental design. For the pilot and the main studies five week old weaning piglets have been vaccinated with tetanus toxoid at days 0 and 14. Time intervals of blood sampling and microarray analyses are indicated by blue arrows. In the main studies anti-TT AB titers have been quantified by ELISA assays for day 28 plasma samples. As juveniles the same animals have been performance-tested, and performance data and AB titers provided a basis for identification of divergent phenotypes for lean growth and humoral immune response, respectively.

1.4 Summary and Aims

The domestic pig is a very important agricultural animal whose economically relevant traits need to be combined in current breeding approaches with emergent societally important traits. One of those economic traits, lean growth, i.e. high proportions of lean meat but low proportions of fat, and one of the societally relevant traits, immune responsiveness are central in the present thesis. Pigs show considerable genetic variation and selection of favourable genotypes seems feasible but little is known about genetically determined dependencies between growth performance and immune responsiveness. Concerning the growing acceptance of the pig as biomedical model organism, the findings in order to further the understanding of lean growth and immune traits relationship may also be of importance for human biology and medicine.

The studies presented in the next chapter attempt to examine responses of PBMCs *in vivo* from divergent phenotypes to immune challenge by genome wide transcriptome analysis. Immune stimulation and mRNA samples were achieved by two vaccination events of antigen-naïve weaning piglets against tetanus toxoid followed by periodical blood sampling, respectively. Initially, a pilot study to evaluate the analysis of mRNA abundances derived from PBMCs by Affymetrix microarrays provided a foundation for refining ongoing work. Subsequently, high lean growth was compared to low lean growth against the background of both high and low anti-TT antibody titers. Correspondingly, these divergent antibody titers were also compared against the background of high and low lean growth, respectively. Each of these comparisons revealed large numbers of differentially expressed genes which were bioinformatically analysed by the assignment to canonical pathways. Eventually, these analyses aimed to identify biofunctions that were differentially activated between divergent phenotypes.

2 Publications

2.1 Summary of publications

A pilot study in order to explore and evaluate microarray analyses of PBMC response to tetanus toxoid vaccination of pigs is described by the first publication (Adler *et al.* 2013a in *PloS one*, see chapter 2.2). It is based on PBMC sampling from multiple tight time intervals throughout two vaccination events and examines the numbers of significantly affected genes and resulting canonical pathways.

Further work was aimed at PBMC transcriptomes of three important time intervals during both vaccinations and a dissection of pig phenotypes that differ for immune response as quantified by anti-tetanus antibody titers and lean growth performance reflected by lean-to-fat parameters, respectively. First, a comparison between four differentiated phenotypes obtained by combination of high and low lean growth and AB titers, respectively, has been reported and revealed high lean growth to be associated with immune activation at the level of transcriptome (Adler *et al.* 2013b in *International journal of biological sciences*, chapter 2.3). Finally, the comparison between phenotype-specific responses to the first and to the second vaccination revealed the adaptive immune response to the second vaccination to reflect considerable differences between divergent lean growth phenotypes and an association between high antibody responses and the enrichment of B cell related signaling within the high lean growth group (Adler *et al.* 2015 in *Physiological genomics*, section 2.4).

2.2 Transcriptomic response of porcine PBMCs to vaccination with tetanus toxoid as a model antigen

Adler, M., Muráni, E., Brunner, R., Ponsuksili, S., & Wimmers, K. (2013).
PloS one, 8(3), e58306.

Content summary:

Based on *in vivo* immune stimulation using tetanus toxoid vaccine as experimental antigen this paper describes microarray analyses of porcine PBMCs (peripheral blood mononuclear cells). Overall, the transcriptome data comprised more than 5000 genes with different transcript abundances. Beside canonical pathways of immune responses several other biofunctions were shown to be affected.

Author contributions:

K. Wimmers, S. Ponsuksili, E. Muráni and R. Brunner conceived and performed vaccination, blood sampling and PBMC preparation for microarray analyses. M. Adler (80 %), S. Ponsuksili and K. Wimmers analysed the microarray data. M. Adler (90 %) and E. Muráni designed and performed RTQ-PCR experiments to confirm the microarray results. M. Adler (90 %) and K. Wimmers wrote the paper.

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PLOS ONE

Transcriptomic Response of Porcine PBMCs to Vaccination with Tetanus Toxoid as a Model Antigen

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Abstract

The aim of the present study was to characterize *in vivo* genome-wide transcriptional responses to immune stimulation in order to get insight into the resulting changes of allocation of resources. Vaccination with tetanus toxoid was used as a model for a mixed Th1 and Th2 immune response in pig. Expression profiles of PBMCs (peripheral blood mononuclear cells) before and at 12 time points over a period of four weeks after initial and booster vaccination at day 14 were studied by use of Affymetrix GeneChip microarrays and Ingenuity Pathway Analysis (IPA). The transcriptome data in total comprised more than 5000 genes with different transcript abundances (DE-genes). Within the single time stages the numbers of DE-genes were between several hundred and more than 1000. Ingenuity Pathway Analysis mainly revealed canonical pathways of cellular immune response and cytokine signaling as well as a broad range of processes in cellular and organismal growth, proliferation and development, cell signaling, biosynthesis and metabolism. Significant changes in the expression profiles of PBMCs already occurred very early after immune stimulation. At two hours after the first vaccination 679 DE-genes corresponding to 110 canonical pathways of cytokine signaling, cellular immune response and other multiple cellular functions were found. Immune competence and global disease resistance are heritable but difficult to measure and to address by breeding. Besides QTL mapping of immune traits gene expression profiling facilitates the detection of functional gene networks and thus functional candidate genes.

Citation: Adler M, Murani E, Brunner R, Ponsuksili S, Wimmers K (2013) Transcriptomic Response of Porcine PBMCs to Vaccination with Tetanus Toxoid as a Model Antigen. PLoS ONE 8(3): e58306. doi:10.1371/journal.pone.0058306

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Introduction

In pig farming, the incidence and severeness of infectious diseases has direct influence on animal welfare, product quality and economics. Since both intensive and organic production systems are faced with multiple infectious diseases, there is a need for animals endogenously protected against a broad range of pathogens.

Sustaining the pig's genetic resistance to infection has been considered as a key breeding goal to improve disease prophylaxis [1]. Wilkie and Mallard [2] proposed indirect selection for general disease resistance to avoid that selection for resistance to specific infections or diseases lead to susceptibility to others.

Little is known about the genetic control within the relationship between immune traits and performance traits. In poultry where several studies are available the selection for high growth rates resulted adversely in an impaired immune competence [3,4]. Vice versa, high immune responsiveness may be associated with a corresponding allocation of resources on the costs of productivity. However, in the pig selection for high immune response was associated with enhanced weight gain [5,6]. Taken together, in order to identify genotypes for selection the investigation of underlying immunogenetic fundamentals plays a major role.

First studies of gene regulation during the porcine immune response focussed on peripheral blood mononuclear cells (PBMCs) so far were limited to candidate genes [7–10]. For the study of gene expression due to immune stimulation or pathogen infection microarray techniques enable the interrogation of large sets of genes [11]. Typical study designs comprise either PBMC cultures stimulated with mitogens [12,13] or the investigation of blood cells or immune tissue after pathogen infection [14–16] or vaccination [17].

Concerning the porcine immune response on the transcriptomic level, our study was addressed to piglets after weaning which often suffer from production diseases caused by various factors including multiple facultative pathogens. To get a genome-wide comprehensive insight into *in vivo* transcriptional changes during the immune response, weaned piglets were vaccinated using tetanus toxoid (TT) as a model antigen for immune stimulation. TT vaccines are known to trigger both the cellular (Th1) and the humoral branch (Th2) of the immune system [18,19] and represent a non-ubiquitous antigen for which weaning piglets are considered as antigen-naïve [17]. Thus, to identify gene transcripts with different abundances and their networks that are altered in leukocytes during the *in vivo* response to a model vaccine inducing a mixed Th1/Th2 response expression profiles of PBMCs before and several time points after immunization are displayed via genome-wide microarrays.

Materials and Methods

Animals, Vaccination and Blood Sampling

Animals used were owned by the Leibniz-Institute for Farm Animal Biology which gave the permission to perform the study. Animal care and tissue collection procedures followed the guidelines of the German Law of Animal Protection and the experimental protocol was approved by the Animal Care Committee of the Leibniz Institute of Farm Animal Biology and the State Mecklenburg-Vorpommern (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei; LALLF M-V/TSD/7221.3-2.1-020/09).

One week after weaning at an age of five weeks three male and three female piglets from three respective litters (n = 18) of a German Landrace herd were vaccinated subcutaneously with 1 ml (30 IU) of tetanus vaccine, composed of TT and aluminium hydroxide as adjuvant (Equilis Tetanus-Vaccine, Intervet, Unterschleißheim, Germany). Directly before (0), as well as 2, 4, 8, 24 and 75 hours after vaccination blood samples were collected (Figure 1). Vaccination and blood sampling were replicated 14 days after the first vaccination, a final blood sample was taken at day 28 when animals of our population reached antibody titers of 0.33 IU/ml with a standard deviation of 0.23 IU/ml.

RNA isolation, Target Preparation and Microarray Hybridization

From 4 ml of blood PBMCs were isolated by centrifugation on a Histopaque (Sigma-Aldrich, Taufkirchen, Germany) density gradient. From the PBMCs preparations total RNA was isolated using TRI reagent (Sigma-Aldrich, Taufkirchen, Germany) followed by DNase treatment and a column based purification using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA integrity was checked by visualization on 1% agarose gel containing ethidium bromide and the concentration was quantified by a NanoDrop ND-1000 spectrometer (PEQLAB, Erlangen, Germany). The absence of DNA contamination was tested by a PCR amplification of the porcine GAPDH gene (forward primer 5′-AAGCAGGGATGATGTTCTGG-3′; reverse primer 5′-ATGCCTCCTGTACCACCAAC-3′). All RNA was stored at -80°C until downstream preparation was performed. For each sampling time point three RNA pools were generated each of six individuals, one male and one female from the three respective litters. Biotin-labeled cRNA was synthesized using the GeneChip 3′ IVT Express Kit (Affymetrix, Santa Clara, CA, USA). Fragmented cRNA was hybridized on Affymetrix GeneChip Porcine Genome Arrays. After staining and washing the arrays were scanned and raw data were obtained using Affymetrix GCOS 1.1.1 software. According to the MIAME standard the microarray data has been deposited in the database of the National Center for Biotechnology Information Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo [GEO: GSE38602].

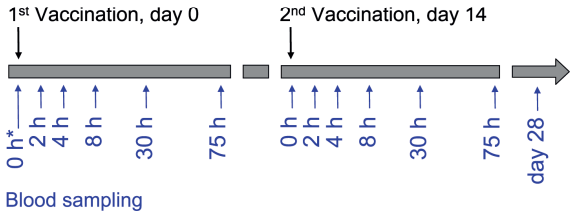

Figure 1. Experimental Design. Animals were vaccinated twice with TT. Directly before (0), as well as 2, 4, 8, 24 and 75 hours after each vaccination blood samples were collected. A final blood sample was taken at day 28. Pairwise comparisons of the reference time 0 hours against each following sampling time point were set up for the identification of DE-genes. * reference time point.
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Data and Pathway Analysis

First, the microarray raw data were quality controlled by the MAS5 and normalized by the PLIER algorithm using Affymetrix Expression Console 1.1 software (Affymetrix, St. Clara, CA, USA). Pairwise comparisons of the three pools of the reference time of 0 hours against the respective following time points after the first and second vaccination were set up (Figure 1). Each comparison was subjected to a variance filter (TM4 Microarray Software Suite, [20,21]) leaving 9000 probe sets of high variance. Afterwards, for each probe set a paired t-test of each comparison was performed. Resulting p values of each comparison were converted to q values, a false discovery rate estimation proposed by Storey and Tibshirani [22]. Bioinformatic analysis of significantly regulated transcripts ($p<0.05$) was carried out by use of a recent annotation of Affymetrix probe sets [23] and the Ingenuity Pathways Analysis (IPA) Software [24].

Quantitative Real-time PCR

For validation of microarray data, the gene expression of five selected genes was determined using the same sample pools of cDNA used for microarray analyses. These cDNA pools were amplified by quantitative real-time PCR on an iQ5 PCR system (Bio-Rad Laboratories, München, Germany). Each amplification was done in duplicate in a final volume of 20 µl with 10 µl of LightCycler 480 SYBR Green I Master (Roche, Mannheim, Germany), 7.7 µl of Aqua dest., 0.4 µl of each primer (10 µM) and 30 ng (1.5 µl) cDNA. The amplified genes were KRAS, RPS6KB1, CD8A, CALR, STAT1, HPRT1 and PPIA (Table 1), while the last two were used as reference genes to account for variation of cDNA amounts after reverse transcription by calculating a normalization factor. Except for KRAS and HPTR1 the genes were amplified by nested PCR. A cDNA standard was amplified with the outer primer pair. The standard curve was derived from amplification of serial dilutions of the standard.

Results

We performed a microarray study of 12 sampling time points over a period of four weeks to get a comprehensive overview of gene expression changes during immune stimulation with TT as a model antigen. The examination of the transcriptomic response was performed with Affymetrix GeneChip Porcine Genome Arrays containing 24,123 probe sets of which 20,689 recently had been assigned to known porcine genes [23]. To analyse transcriptional changes pairwise comparisons were set-up of each time point after vaccination against 0 hours serving as the reference. Subsequently, by use of the IPA database for each time point after immunization canonical pathways were identified, which represented a significant number of genes with different transcript abundances (for simplicity, hereinafter referred to DE-genes).

DE-genes after Tetanus Vaccination and Assignment to Canonical Pathways

To assign probesets to genes the microarray data of each comparison were submitted to the manually curated database Ingenuity Pathway Analysis [24]. The transcriptome data in total comprised more than 5000 DE-genes ($p<0.05$). Within the single time stages the numbers of DE-genes were between several

Table 1. Primer sequences used for quantitative real-time PCR.

Gene	Probe set ID	Outer primer sequence 5′-3′	Inner primer sequence 5′-3′
KRAS	Ssc.29092.1.A1_at	–	For TTCGTGTTCCCTCAATGTTTC Rev TGGTGCATGCAGTCAATTACT
RPS6KB1	Ssc.22127.1.S1_at	For TGATGAATGTCTTCCACAGTGA Rev GGAGAACATAGCAAGCAGCA	For CCTGCCTTAAAGAGCATTTCC Rev CGCACACTCAGACTGAAGACA
CD8A	Ssc.23489.1.S1_at	For CTGAATCCTGGAAAGTGAACAA Rev TCGGTCATAATTCTGTGTTTACAA	For CACGACCTCTAAAGGAAATCCA Rev CGAGGAGCACGTTCAAATATC
CALR	Ssc.3106.1.S1_at	For GGAGTTTGGCAACGAGACAT Rev AGGAATCTGGGGAGAGGAGA	For AGGCCAAGGATGAGCTGTAG Rev ACCAAATCCATCCCAAATCA
STAT1	Ssc.6025.2.A1_at	For CGGGGCATAAAAGTTGTGTT Rev CGGTTTCTCCTCAGTTTTGAA	For GGCTTTATGCTGCTGGCTAC Rev CTGGCTCCCTTGATAGAACTG
HPRT1*	Ssc.4158.1.S1_at	–	For GTGATAGATCCATTCCTATGACTGTAGA Rev TGAGAGATCATCTCCACCAATTACTT
PPIA*	Ssc.8046.1.A1_at	For AGCACTGGGGAGAAAGGATT Rev TGTCCACAGTCAGCAATGGT	For GATTTATGTGCCAGGGTGGT Rev CTTGGCAGTGCAAATGAAAA

*reference genes used for normalization.

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hundred and more than 1000 with different proportions of up and down regulation (Table 2). IPA canonical pathways were identified for DE-genes ($p<0.05$, fold change >1.3) of each pairwise comparison of 0 hours to the respective time points after initial and second vaccination.

Here, we examined the transcriptional response at four time points within one day after each vaccination and the later response at 75 h and day 14. Concerning the former, i.e. the early response from two to 24 hours after vaccination, a top 20 list of canonical pathways was set-up derived by rank sums over the respective four time points (Tables 3 and 4). Based on individual rankings of pathways within the respective time points the rank sum represents the top pathways, which were present either repeatedly during day one or predominantly at single time points.

Table 2. Number, direction and q-values [34] of DE-genes ($p<0.05$) over the time points of blood sampling after vaccination.

Time after 1st vaccination	2 h	4 h	8 h	24 h	75 h	d 14
Number of DE-genes	679	1196	1104	773	549	642
increased transcript abundance	417	1063	889	233	118	207
decreased transcript abundance	262	133	215	540	431	435
q-value (at p = 0.05)	0.24	0.08	0.08	0.22	0.36	0.21
Time after 2nd vaccination	**2 h**	**4 h**	**8 h**	**24 h**	**75 h**	**d 28**
Number of DE-genes	485	358	824	942	1121	527
increased transcript abundance	301	187	406	557	259	408
decreased transcript abundance	184	171	418	385	862	119
q-value (at p = 0.05)	0.49	0.59	0.24	0.15	0.09	0.32

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First Vaccination

Significant changes in the trancript abundances of PBMCs already occurred very early after *in vivo* immune stimulation. At 2 h after vaccination 679 DE-genes corresponding to 110 canonical pathways of immune response and other multiple cellular functions were found (see Dataset S1).

A considerable high number of transcripts with different abundance has been found at 4 h (1196 DE-genes) and 8 h (1104 DE-genes), that could be assigned to 72 and 99 canonical pathways, respectively. At 24 h after the first immunization 773 DE-genes were found related to 148 canonical pathways.

Concerning these four time points, i.e. within one day after vaccination, signaling pathways of multiple biological function were present (Table 3). Pathways of 'cellular immune response', 'cellular growth, proliferation and development' and 'intracellular and second messenger signaling' as well as 'organismal growth and development' were predominant (Figure 2).

75 h after the first vaccination for 118 increasingly- and 431 decreasingly abundant DE-genes a number of 37 canonical pathways were found. Pathways of the categories 'cellular' and 'organismal growth and development' as well as 'intracellular and second messenger signaling' were predominant.

At 14 days after the first vaccination, i.e. directly before the second vaccination, 207 increasingly abundant and 435 decreasingly abundant DE-genes were present. These genes correspond to 53 canonical pathways mostly represented by 'cellular immune response' or 'cytokine signaling' processes and 'intracellular and second messenger signaling' as well.

Second Vaccination

2 h after the second vaccination 485 DE-genes were observed related to 19 canonical pathways, at 4 h 358 DE-genes correspond to 27 affected canonical pathways. The number of DE-genes gained to 824, 942 and 1121 at 8 h, 24 h and 75 h respectively. These genes were found associated with 155, 97 and 179 canonical pathways respectively, mostly represented by 'cellular immune response', 'cellular' and 'organismal growth and development' as well as 'intracellular and second messenger signaling' (Table 4, Figure 2).

For the final sampling 14 days after the second vaccination the numbers of DE-genes were 408 for increasingly abundant and 119 for decreasingly abundant related to 39 canonical pathways with

Table 3. Top 20 canonical pathways within the first 24 h of immune response after the first vaccination.

Ingenuity Canonical Pathway	Pathway Category	−log p at 2 h	−log p at 4 h	−log p at 8 h	−log p at 24 h
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	1	6.79	2.25	2.37	6
CD28 Signaling in T Helper Cells	1	4.92	n.s.	1.68	5.63
CTLA4 Signaling in Cytotoxic T Lymphocytes	1	3.64	n.s.	n.s.	6.06
T Cell Receptor Signaling	1	2.2	n.s.	3.22	5.79
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	1; 2	1.48	n.s.	4.13	2.31
Clathrin-mediated Endocytosis Signaling	1; 3; 9	4.15	2.66	n.s.	4.63
ILK Signaling	4	4.69	2.03	n.s.	1.59
FAK Signaling	4	3.83	1.58	1.68	4
VEGF Signaling	4; 10	3.11	2.98	2.6	4.74
IGF-1 Signaling	4; 10	1.61	3.5	2.63	3.54
Integrin Signaling	4; 5; 7	6.2	2.74	2.94	4.08
Protein Ubiquitination Pathway	5	1.45	3.7	5.69	n.s.
ERK/MAPK Signaling	5	3.01	2.05	4.26	5.09
Glucocorticoid Receptor Signaling	5	2.59	2.95	4.76	3.65
Estrogen Receptor Signaling	6	n.s.	3.82	4.02	n.s.
Aldosterone Signaling in Epithelial Cells	6; 12	2.6	2.47	3.06	2.12
NRF2-mediated Oxidative Stress Response	8; 13	2.11	2.14	5.93	1.66
Actin Cytoskeleton Signaling	9	5.15	3.11	4.62	4.81
Regulation of Actin-based Motility by Rho	11	3.94	3.68	1.36	1.62
Ephrin Receptor Signaling	9	2.71	n.s.	n.s.	5.45

1 Cellular Immune Response.
2 Cytokine Signaling.
3 Pathogen-Influenced Signaling.
4 Cellular Growth, Proliferation and Development.
5 Intracellular and Second Messenger Signaling.
6 Nuclear Receptor Signaling.
7 Cell Cycle Regulation.
8 Cellular Stress and Injury.
9 Organismal Growth and Development.
10 Growth Factor Signaling.
11 Neurotransmitters and Other Nervous System Signaling.
12 Cardiovascular Signaling.
13 Ingenuity Toxicity List Pathways.
n.s. not significant.
doi:10.1371/journal.pone.0058306.t003

predominant functions in immune response and 'cellular growth, proliferation and development'.

Certain pathways were found to be affected over nearly all time points including integrin, VEGF, actin cytoskeleton and glucocorticoid receptor signaling. The transcriptional responses at four time points at day one after each vaccination are summarized in Tables 3 and 4. A comparison of the most frequently observed categories of these canonical pathways is illustrated by Figure 2. For both vaccinations canonical pathways of immune response were predominant followed by 'cellular growth, proliferation and development' and 'intracellular and second messenger signaling'. For the second vaccination among the top ranking also pathways of apoptosis and metabolism are present.

Validation by Quantitative Real-time PCR

For five selected genes the expression values of the microarray data were validated by comparison to copy numbers determined by quantitative real-time PCR. Relative transcript abundance of three genes were significantly correlated at 0.42 to 0.58; for two genes a moderate correlation at 0.35 to 0.36 was observed (Table 5).

Discussion

The objective of this study was to evaluate global transcriptional *in vivo* response of porcine PBMCs to immune stimulation using TT as a T cell dependent model antigen triggering a mixed Th1/Th2 immune reaction. Our results revealed a large number of genes with differential transcript abundance. The bioinformatic analysis of these genes via the ingenuity knowledge base displayed differential levels of transcripts assigned to numerous canonical pathways. Among these, with regard to immune function, 'cellular immune response' and 'cytokine signaling' pathways were predominant. In terms of further functions the activity of PBMCs was mainly made up of 'cellular growth, proliferation and development', 'intracellular and second messenger signaling' as well as 'organismal growth and development'.

Our microarray data show that *in vivo* already very early after TT vaccination, i.e. at 2 h, 4 h and 8 h, considerable changes on

Table 4. Top 20 canonical pathways within 24 h of immune response after the second vaccination on day 14.

Ingenuity Canonical Pathway	Pathway Category	−log p at 2 h	−log p at 4 h	−log p at 8 h	−log p at 24 h
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	1	n.s.	n.s.	3.77	3.36
fMLP Signaling in Neutrophils	1; 3	n.s.	n.s.	3.8	2.82
Clathrin-mediated Endocytosis Signaling	1; 4; 10	1.69	n.s.	4.88	2.78
Tumoricidal Function of Hepatic Natural Killer Cells	1; 6	1.64	1.54	n.s.	n.s.
B Cell Receptor Signaling	2	n.s.	n.s.	3.18	3.23
Role of MAPK Signaling in the Pathogenesis of Influenza	4; 5	1.62	1.48	1.84	1.63
Atherosclerosis Signaling	5; 13	n.s.	2.39	n.s.	n.s.
SAPK/JNK Signaling	6	n.s.	n.s.	5.84	2.75
Integrin Signaling	7; 8; 9	2.46	2.15	3.69	3.59
ILK Signaling	7	n.s.	n.s.	5.17	1.84
VEGF Signaling	7; 11	n.s.	n.s.	4.04	4.27
Protein Kinase A Signaling	8	n.s.	3.74	4.31	n.s.
Glucocorticoid Receptor Signaling	8	n.s.	n.s.	2.37	3.79
Insulin Receptor Signaling	8	2.05	n.s.	1.65	1.77
Actin Cytoskeleton Signaling	10	2.3	n.s.	5.37	5.16
Ephrin Receptor Signaling	10	2.15	1.35	1.66	n.s.
Regulation of Actin-based Motility by Rho	12	1.31	n.s.	2.87	3.13
Inhibition of Angiogenesis by TSP1	13	2.36	n.s.	3.21	n.s.
Aminosugars Metabolism	14	n.s.	2.9	n.s.	n.s.
N-Glycan Degradation	15	n.s.	2.48	n.s.	n.s.

1 Cellular Immune Response.
2 Humoral Immune Response.
3 Cytokine Signaling.
4 Pathogen-Influenced Signaling.
5 Disease-Specific Pathways.
6 Apoptosis.
7 Cellular Growth, Proliferation and Development.
8 Intracellular and Second Messenger Signaling.
9 Cell Cycle Regulation.
10 Organismal Growth and Development.
11 Growth Factor Signaling.
12 Neurotransmitters and Other Nervous System Signaling.
13 Cardiovascular Signaling.
14 Carbohydrate Metabolism.
15 Glycan Biosynthesis and Metabolism.
n.s. not significant.
doi:10.1371/journal.pone.0058306.t004

the mRNA level occurred. Using a cDNA expression array with 588 genes Regnström and colleagues observed an immediate transcriptional response of murine spleen cells already 4 h after *in vitro* restimulation by TT covering Th1 and Th2 markers. [25]. *In vivo*, we observed an even earlier broad transcriptional response of naïve PBMC. Among the Top 20 canonical pathways at day one after the initial vaccination (Table 3) the signaling pathway 'regulation of IL-2 expression in activated and anergic T lymphocytes' indicates the early occurence of cytokine signaling events. In addition, IL-2 signaling and other cytokine pathways, IL-3, IL-4 and IFNγ signaling were found among the predominant pathways following each vaccination. The T cell growth factor IL-2 is known to induce the proliferation of T cells autocrinally as well as the proliferation of B cells. Produced by activated T cells it is the most important cytokine for the development of adaptive immune responses. IL-2 pathways featured almost up shifted transcripts within day one after the initial vaccination. At 8 h after the first vaccination as key molecules of these pathways we found increasead transcript abundance for the CD3 receptor and the ELK1 transcription factor for IL-2 expression (see Dataset S1).

Likewise, several T cell costimulatory pathways in particular 'CD28 signaling in T helper cells', 'CTLA4 signaling in cytotoxic T lymphocytes' and 'T cell receptor (TCR) signaling' showed a considerable response early at 2 h, 8 h and 24 h (Table 3) as well as moderate responding after the second vaccination (see Dataset S1). T cell receptor (TCR) signaling is responsible for signal transduction after MHC associated antigenes are recognized and bound by the TCR. Following the first vaccination we found transcripts of the costimulatory receptors CD4 increased and CD28 decreased (both at 2 h) and for the CD3 receptor increased (at 8 h) which is part of the TCR-CD3 complex. The binding of antigens to TCR leads to a sequence of tyrosinase activity eventually resulting in transcriptional activation of several genes. For one of these downstream signaling processes we found increased transcript abundance of NFAT, a transcription factor of the IL-2 gene at 24 h after the second vaccination.

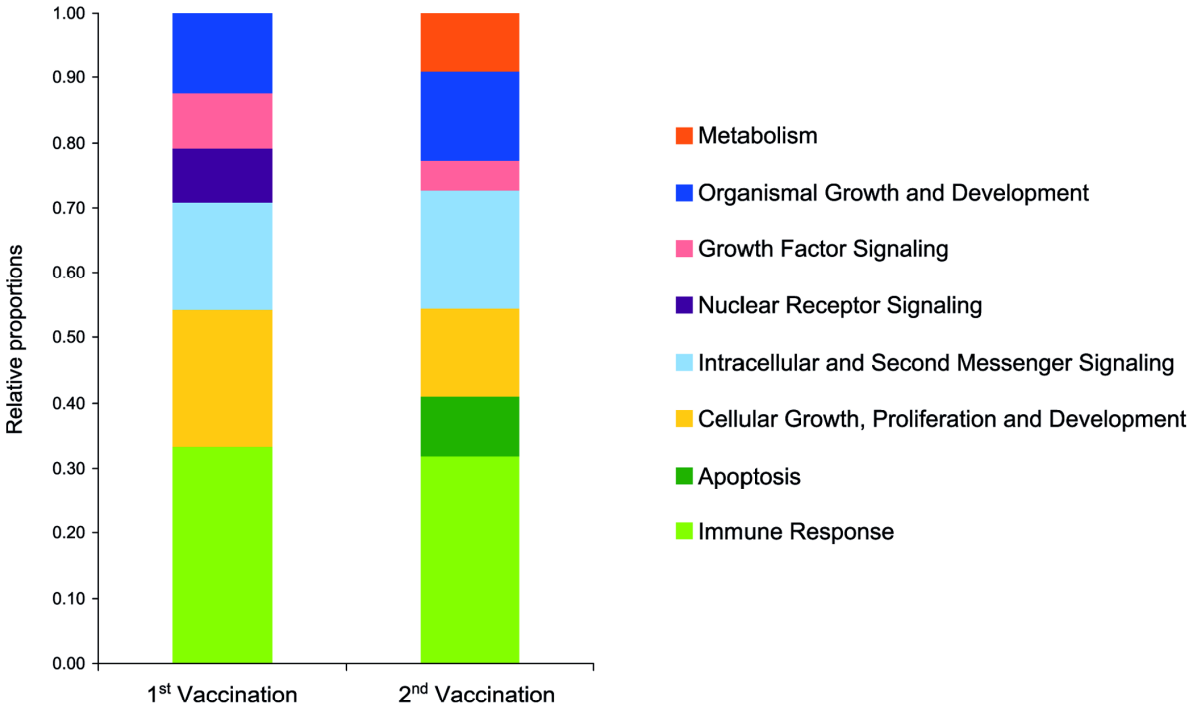

Figure 2. Most affected biofunctions within 24 hours after the first and after the second vaccination. Segments of the respective bars for the first and the second vaccination represent the relative frequencies of IPA biofunctional categories superior to canonical pathways that are most significant at 2 h, 4 h, 8 h and 24 h after immunization.
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The costimulatory pathways 'CD28 signaling in T helper cells' and 'CTLA4 signaling in cytotoxic T lymphocytes' generally represent antagonistic processes in T cells. CD28 acts as a positive costimulatory receptor for B7 molecules on antigen presenting cells whereas CTLA4, which is also a receptor for B7, is known to alter costimulatory to inhibitory signals. However, we did not find changes of transcript quantities of CTLA4 and associated negative-signaling proteins at the early time points rather decreased transcript abundance after the second vaccination of SHP1 and CTLA4 at 8 h and 24 h, respectively.

For CD28 Signaling we hardly found a conclusive alteration of pathway components at early time points, except for transcript increase of calmodulin, calcineurin and NFAT (activated by calmodulin-calcineurin interaction) indicating activated IL-2 transcription at 24 h after second vaccination. In addition, we observed an obvious down regulation at day three (75 h - second vaccination) with ten molecules of decreased transcript abundance.

In our results based on Ingenuity Pathway Analysis the response of PBMCs was mainly made up of cellular immune response and cytokine signaling. Signaling pathways of the humoral immune response were not found among the predominant. However, it should be mentioned here that the ingenuity category for humoral immune response listed only 17, whereas the category cellular immune response listed 70 canonical pathways with some overlap between the two groups. Consequently, the category humoral immune response may appear under-represented, although a number of DE-genes involved in humoral immune events were found. For instance the humoral immune response pathway 'B Cell Receptor Signaling' was found regulated at six time points.

TT vaccines are known to trigger a mixed Th1 and Th2 immune response [18,19] and represent a non-ubiquitous antigen which has been used as model antigen here. To stimulate an *in vivo* immune response in pig, tetanus vaccine was administered to piglets. In general, in addition to the toxoid, vaccines contain

Table 5. Correlation between microarray gene expression and results of quantitative real-time PCR for selected genes.

Gene	Spearman's rho	p-value	Number of involved canonical pathways
KRAS	0.58	<0.01	92
RPS6KB1	0.58	<0.01	20
CD8A	0.35	0.07	3
CALR	0.36	0.06	4
STAT1	0.42	0.04	26

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aluminum adjuvants generally known as immunostimulators for the Th2 type of immune response [26]. For tetanus vaccination it has been shown that it is only the combination of TT and adjuvant, which causes an effective immune response [25,27,28].

Consistent with our findings are earlier results for the pig [17,29] and studies of murine spleen lumphocyctes [25,28] that found differential expression of genes involved in both immune response and processes of cell signaling, cellular and organismal growth, cell cycle control, apoptosis, cytoskeleton organization, biosynthesis, metabolism as well as stress, toxicology response and oncogenesis or tumor suppression.

Moreover, our microarray results and the aforesaid studies in mice demonstrate clearly an early onset of transcriptional responses to immune stimulation within few hours that had also been shown for murine T cell stimulation with superantigen [30]. However, we also found evidence that the early response after the second vaccination was less pronounced given the fact that only few and hardly any immune specific canonical pathways could be found at 2 h after the booster vaccination. Further, we observed indications that the response to the second vaccination was prolonged in terms of the duration of shifts of transcript abundance. In fact, pathways found at 75 h after the initial vaccination were hardly found among the pathways affected during the first 24 h. However, after the booster vaccination the time point 75 h shared multiple pathways with the previous samplings.

Disease resistance and immune competence are heritable [31,32] but difficult to measure and hence to address by breeding. QTL mapping of cellular and humoral immune traits [33–35] in experimental cross breeds facilitates the identification of candidate genes for immune competence. Recently QTL for TT antibodies were detected on SSC 2, 4, 8, 11 and 18 [36]. Beside the mapping approach gene expression profiling of immune tissues in defined phenotypes enables the detection of functional networks and thus functional candidate genes. Concerning both approaches additional research is needed to further characterize these candidate genes to reveal genetic markers for selection of animals endogenously better protected against infection.

Supporting Information

Dataset S1 Significant IPA canonical pathways and respective DE-genes ($p<0.05$, FC >1.3). Excel file with 12 spreadsheets for 12 time points after vaccination and one spreadsheet listing DE-genes over all time points.
(XLS)

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Author Contributions

Conceived and designed the experiments: KW SP EM. Performed the experiments: KW MA SP EM RB. Analyzed the data: MA SP KW. Contributed reagents/materials/analysis tools: KW SP EM RB. Wrote the paper: MA KW.

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2.3 PBMC transcription profiles of pigs with divergent humoral immune responses and lean growth performance

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Content summary:

This study compares longitudinal expression profiles of PBMCs from domestic pigs with divergent phenotypes for lean growth (LG) and anti-TT antibody (AB) titers, respectively. The comparison of high and low LG predicted a higher activation state in high LG animals for immune response pathways related mainly to T cell action and antigen presentation. However, high AB, compared to low AB, had decreased transcript abundances in the most significant pathways that where predominantly related either to T cell mediated responses (if combined with low LG) or to other biofunctions than lymphocyte-related immune responses (if combined with high LG).

Author contributions:

K. Wimmers, M. Adler (30 %), S. Ponsuksili and E. Muráni developed the concept of phenotype differentiation. M. Adler (50 %) and K. Wimmers performed vaccination, blood sampling and PBMC preparation. M. Adler (100 %) evaluated and performed the Anti-TT antibody ELISA assays. M. Adler (80 %) and K. Wimmers collected phenotype data of productive traits and performed the principal component analysis. M. Adler (100 %) isolated RNA samples from PBMC preparations and selected the animals for microarray analyses. M. Adler (90 %) and K. Wimmers analysed the microarray data. M. Adler (90 %) and K. Wimmers wrote the paper.

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Research Paper

PBMC Transcription Profiles of Pigs with Divergent Humoral Immune Responses and Lean Growth Performance

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Abstract

Background: The identification of key genes and regulatory networks in the transcriptomic responses of blood cells to antigen stimulation could facilitate the understanding of host defence and disease resistance. Moreover, genetic relationships between immunocompetence and the expression of other phenotypes, such as those of metabolic interest, are debated but incompletely understood in farm animals. Both positive and negative associations between immune responsiveness and performance traits such as weight gain or lean growth have been reported.

We designed an *in vivo* microarray study of transcriptional changes in porcine peripheral blood mononuclear cells (PBMCs) during the immune response to tetanus toxoid (TT) as a model antigen for combined cellular (Th1) and humoral (Th2) responses. The aim of the study was to investigate the responsiveness of PBMCs against the background of divergent lean growth (LG) performance and anti-TT antibody (AB) titers and to compare lean growth and humoral immune performance phenotypes.

Results: In general, high LG phenotypes had increased cellular immune response transcripts, while low AB phenotypes had increased transcripts for canonical pathways that represented processes of intracellular and second messenger signaling and immune responses. Comparison of lean growth phenotypes in the context of high AB titers revealed higher cellular immune response transcripts in high LG phenotypes. Similar comparisons in the context of low AB titers failed to identify any corresponding pathways. When high and low AB titer phenotypes were differentially compared, low AB phenotypes had higher cellular immune response transcripts on a low LG background and higher cell signaling, growth, and proliferation transcripts on a high LG background.

Conclusions: Divergent phenotypes of both lean growth performance and humoral immune response are affected by significant and functional transcript abundance changes throughout the immune response. The selected high-performance phenotypes demonstrated both high AB titers and increased transcript abundance of cellular immune response genes, which were possibly offset by lower expression of other cellular functions. Further, indications of compensatory effects were observed between cellular and humoral immune responses that became visible only in low-performance phenotypes.

Key words: leukocyte; porcine; microarray; pathway analysis; tetanus vaccination

Introduction

Animal health and welfare dictates livestock quality and the practice of economically efficient farming; in turn, animal health relies on the prevention of infectious diseases. Therefore, the genetic fundamentals of immunocompetence, or immunogenetics, have become a central research subject in farm

animals. Moreover, strong selection for production traits is presumed to have led to impairments concerning behavior, physiology and immune function [1,2]. Hence, comprehensive selection approaches for traits of disease resistance and immunocompetence are proposed [3-6]. However, the relationship between performance traits and immunocompetence, genetic fundamentals, and impacts on breeding are not well understood. Concerning the direct relationship between immune responsiveness and performance, earlier porcine studies report a negative correlation between post-vaccination antibody titers and weight gain [7]. Conversely, other studies report that selection for high immune response associates with enhanced weight gain [8,9]. Clapperton and colleagues [10] show that selection for lean growth associates with higher numbers of several types of lymphocytes and monocytes in Large White pigs.

Transcriptome profiling of peripheral blood mononuclear cells (PBMCs) throughout immune responses is widely used to identify the extent and kinetics of differential gene expression [11-16]. Identification of affected signaling and metabolic pathways and their key genes allows better understanding of host defence and disease resistance. Moreover, the PBMC transcriptome represents not only the primary immune function of leukocytes, but also displays transcriptomic shifts of other tissues and organs due to physiological and environmental alterations [17-19]. Immune-stimulated physiological and metabolic changes in other tissues may also manifest through PBMC gene expression, however, this remains to be confirmed.

Previously, we evaluated a microarray study of *in vivo* transcriptional changes in porcine PBMCs during the immune response to tetanus toxoid (TT) as a model antigen for a combined immune response [16]. TT vaccines trigger both the cellular (Th1) and humoral (Th2) branches of the immune system [20,21] and represent a non-ubiquitous antigen for which weaning piglets are antigen-naïve [11]. To gain further insight into the relationship between immunocompetence and performance, here we compared the responsiveness of PBMCs to TT vaccination against the background of divergent lean growth performance and anti-TT antibody (AB) titers, respectively. Analyses of affected canonical pathways and downstream effects were compared between divergent phenotypes for lean growth performance and humoral immune response.

Material and methods

Animals, vaccination, sampling and phenotype recording

The Leibniz-Institute for Farm Animal Biology provided animals and permission for this study. Animal care, vaccination, and blood collection were performed according to the guidelines of the German Law of Animal Protection. The experimental protocol was approved by the Animal Care Committee of the Leibniz Institute of Farm Animal Biology and the State Mecklenburg-Vorpommern (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei; LALLF M-V/TSD/7221.3-2.1-020/09).

The experimental design is outlined in Fig. 1. One week after weaning, 160 five-week-old German Landrace piglets of a commercial herdbook herd were initially vaccinated (day 0) subcutaneously with one dose (1 mL) of tetanus vaccine, composed of TT and aluminium hydroxide as adjuvant (Equilis Tetanus-Vaccine, Intervet, Unterschleißheim, Germany). After two weeks, a booster vaccination was given (day 14). EDTA blood samples were collected directly before the first (day 0) and second vaccination (day 14), as well as two weeks after the booster (day 28). Plasma samples were prepared and stored at -80°C until further analysis. Plasma anti-TT AB titers were determined in triplicate using a commercially available ELISA (RE57441, IBL International, Hamburg, Germany) according to manufacturer's protocol.

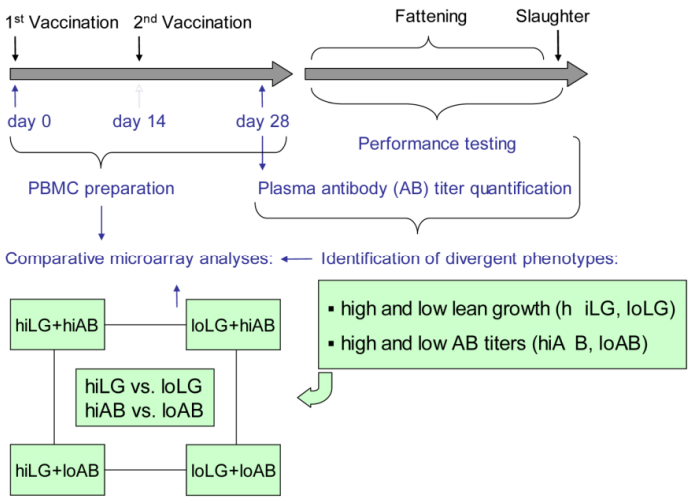

Figure 1. Experimental design. Five-week-old piglets were vaccinated twice with TT. Directly before the first (day 0) and the second (day 14) vaccination as well at day 28 blood samples were collected and PBMCs were isolated. Anti-TT antibody titers were quantified by ELISA from day 28 plasma samples. Animals were then performance-tested. Performance data and AB titers were interpreted as a basis for the identification of divergent phenotypes of lean growth performance and humoral immune response, respectively. Finally, expression profiles of divergent phenotypes for lean growth and humoral immune performance were compared.

Juvenile animals (average age of 10 weeks) were performance tested and evaluated during fattening and at slaughter (final average weight of 110 kg). Performance test data were subjected to a principal component analysis, which identified the first component (factor 1) explaining 39% of the total variance (Supplementary Table 1). Among the animals there was no evidence for association between performance and immune traits. In fact, coefficients of correlation between performance traits and plasma anti-TT AB titers were low ($r \leq 0.1$), thus facilitating the selection of animals that represent divergent groups for both traits. Key parameters (loads) of factor 1 (Table 1) were taken as a basis for the identification of lean growth performance phenotypes. For final phenotype rating, animals from the respective terciles of highest and lowest factor 1 values were categorized as high (hiLG) and low (loLG) lean growth performance, respectively. Accordingly, animals assigned to the first and the third terciles of TT-AB titers were rated as high (hiAB) and low humoral immune response (loAB) phenotypes, respectively.

Finally, ten animals each were selected being representatives of one of the two terciles for lean growth performance and of one of the two terciles for antibody response, respectively. The four groups that were set up for subsequent analyses were balanced for sex and litter.

Table 1. Key traits used for phenotype rating of high lean growth and low lean growth performance.

Trait	High lean growth		Low lean growth		High *vs.* low
	Mean	SD	Mean	SD	p-value
Average backfat (cm)	1.86	0.21	2.44	0.35	5.93 x E-07
Fat area (cm^2)	14.9	2.8	21.5	3.8	3.66E-07
Loin eye area (cm^2)	53.0	4.4	48.0	6.2	6.34E-03
Meat to fat ratio	3.7	0.9	2.3	0.6	1.55E-06
Lean mean content (%)	59.9	1.9	54.6	3.7	4.75E-06

RNA preparation and microarray hybridization

PBMCs were isolated from 4-mL blood samples by centrifugation on a Histopaque density gradient (Sigma-Aldrich, Taufkirchen, Germany). PBMC preparations were stored at -80°C. Total RNA was isolated using Qiazol reagent (Qiagen, Hilden, Germany), then treated with DNase and column-purified using the RNeasy Mini Kit (Qiagen). RNA integrity was visualized by separation on a 1% agarose gel containing ethidium bromide and the concentration was quantified by a NanoDrop ND-1000 spectrometer (PEQLAB, Erlangen, Germany). DNA contamination was assessed by PCR amplification of the porcine GAPDH gene (forward primer: 5′-AAGCAGG GATGATGTTCTGG-3′; reverse primer: 5′-ATGCCTCCTGTACCACCAAC-3′). All RNA was stored at -80°C.

Each RNA sample was transcribed to DNA using the Ambion WT Expression Kit (Ambion, Austin, TX, USA). DNA preparations were fragmented and labelled with the WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA). Labelled DNA was hybridized on snowball arrays, a recently developed and annotated whole genome array [22,23]. Further information about this platform is also available in the 'ArrayExpress' database (accession number A-AFFY-189, http://www.ebi.ac.uk/arrayexpress/arrays/A-AFFY-189/). Following staining and washing, arrays were scanned and raw data generated using Affymetrix GCOS 1.1.1 software. Microarray data is MIAME-compliant and was deposited in the database of the National Center for Biotechnology Information Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo [GEO: GSE47845].

Microarray data processing and functional analyses

Quality-controlled raw data were normalized by the PLIER algorithm using Affymetrix Expression Console 1.1 software (Affymetrix) and filtered by standard deviation ($s \leq 0.2$). According to the experimental design aiming at showing contrasts of the PBMC transcription due to antibody responsiveness and lean growth performance of the test subjects the factor of immune response group (`IR´) and lean growth performance group (`P´) were used as fixed effects in the analysis of variance. Since the genetic background and the age at sampling and expression profiling affect the gene activity `sire´ (`S´) and `time´ (`T´) were also fitted as fixed effects in a mixed model. So essentially, relative transcript abundance changes were determined using these fixed effects and the interactions between `IR´ and `P´ and between `T´, `IR´, and `P´ [$y = \mu + S + T + IR + P + (IR \times P) + (T \times IR \times P) + \varepsilon$]. The mixed model was combined with a repeated statement for the time component in order to take into account correlations among measurements made on the same subject by specifying heterogeneous covariance structure.

Thus sampling time points were weighted equally while comparing lean growth and humoral immune response phenotypes. Mixed model analyses were performed with JMP Genomics 5.0 software (SAS Institute, Cary, NC, USA).

Annotation data for the snowball arrays were obtained from the developers [22,23]. Transcripts

showing different abundance at nominal p-values < 0.5 were assigned to annotated genes and bioinformatically analysed with Ingenuity Pathways Analysis (IPA) Software [24]. At the levels of IPA's functional analyses for canonical pathways and biofunctions a correction for multiple testing was applied by use of IPA's B-H p-value correction procedure. Cut-off criteria were set to corrected p-values < 0.05 for canonical pathways and for biofunctions, respectively, and absolute values of activation z-scores > 2.0 for biofunctions.

Results

Antibody titer and performance characterization

In order to analyse different transcript abundance of PBMC due to immune responsiveness and lean growth performance five-week-old German Landrace piglets were initially vaccinated (day 0) with TT, and a booster vaccination was given on day 14. Animals were then performance-tested until a final average weight of 110 kg at slaughter. Animals of the tails of the distribution for the trait AB titer against TT and lean growth performance were selected. Plasma anti-TT AB titers, determined by enzyme-linked immunosorbent assay (ELISA) at two weeks after booster vaccination, ranged from <0.1 IU/mL to >1.0 IU/mL (mean = 0.33 IU/mL; standard deviation = 0.23 IU/mL). AB titers were interpreted as a basis for the identification of divergent phenotypes of high humoral immune response (hiAB) and low humoral immune response (loAB) and differed significantly among both groups (hiAB: mean = 0.57 IU/mL; standard deviation = 0.13 IU/mL; loAB: mean = 0.23 IU/mL; standard deviation = 0.04 IU/mL; $p < 0.001$). Lean growth performance phenotypes were obtained by the first component of a principal component analysis of extensive performance data. Highly significant differences for key parameters of this first component were used for phenotype rating of high lean growth (hiLG) and low lean growth (loLG) performance (Table 1).

Differentially-expressed genes and assigned functions for divergent lean growth performance phenotypes

Using Ingenuity Pathways Analysis (IPA) software [24], genes with significantly different transcript abundances (hereafter referred to as DE-genes) were assigned to affected canonical pathways and altered downstream effects (hereafter referred to as biofunctions).

Comparison of hiLG and loLG revealed 574 DE-genes (Table 2) that were assigned to pathways predominated by the cellular immune response (Table 4a). The most significant pathways, including T-cell receptor, PKCθ, and CD28 signaling, are responsible for co-stimulation during lymphocyte activation and showed higher transcript abundances in the hiLG group. Functional analysis predicted decreased organismal death and increased development, differentiation, and homeostasis of lymphocytes for the hiLG phenotype (Table 5a).

DE-genes and assigned functions for divergent humoral immune response phenotypes

Comparison of hiAB and loAB revealed 522 DE-genes (Table 2). The loAB group showed higher transcript abundances for canonical pathways (Table 4b) that represented processes of intracellular and second messenger signaling (integrin signaling, phospholipase C signaling) and immune responses (clathrin-mediated endocytosis signaling, IL-8 signaling). Functional analyses predicted decreased cell adhesion, aggregation, proliferation, and differentiation in hiAB phenotypes (Table 5b).

Differentiated comparisons between divergent lean growth performance and humoral immune response phenotypes

The identification of hiLG, loLG, hiAB, and loAB phenotypes enabled differentiated comparisons between hiLG and loLG against backgrounds of hiAB and loAB, respectively, and vice versa: hiLG+hiAB *vs.* loLG+hiAB; hiLG+loAB *vs.* loLG+loAB; hiLG+hiAB *vs.* hiLG+loAB; and loLG+hiAB *vs.* loLG+loAB (Fig. 1). Each comparison revealed more than 400 DE-genes (Table 3).

Similar to the comparison between hiLG and loLG, the hiLG+hiAB *vs.* loLG+hiAB comparison revealed higher transcript abundances in the hiLG+hiAB phenotype that affected pathways assigned exclusively to cellular immune responses (Fig. 2). Correspondingly, downstream effects analysis predicted activation of lymphocyte development, differentiation, and homeostasis and decreased leukocyte apoptosis in hiLG+hiAB (Fig. 3). Although the hiLG+loAB *vs.* loLG+loAB comparison represented 441 DE-genes, no corresponding pathway or biofunction was identified.

When high and low AB titer phenotypes were differentially compared, higher trancript abundances were found for loLG+loAB compared to the loLG+hiAB phenotype. These DE-genes were assigned to canonical pathways of the cellular immune response (Fig. 2) and to an increase of biofunctions such as activation and differentiation of lymphocytes, particularly T lymphocytes, and differentiation of helper T cells (Fig. 3).

The comparison of divergent AB titer phenotypes with high lean growth, hiLG+hiAB *vs.* hiLG+loAB, revealed pathways with cell signaling, growth, and proliferation functions, rather than distinct immune responses (Fig. 2). Transcript abundances were higher in the hiLG+loAB group and predicted activation of cell transformation and spreading, survival, aggregation, adhesion, and binding (Fig. 3).

Table 2. Numbers of differentially-expressed (DE-) genes from comparison of lean growth performance and antibody titers

	Main phenotype comparisons	
	Lean growth performance: high *vs.* low	Antibody titer: high *vs.* low
Number of DE-genes	574	522
Transcript Abundance >	366	133
Transcript Abundance <	208	389

Table 3. Numbers of DE-genes from differentiated comparisons between lean growth performance and antibody titers

	Differentiated comparisons for high (hi) and low (lo) lean growth (LG) and antibody (AB) phenotypes			
	hiLG+hiAB *vs.* loLG+hiAB	hiLG+hiAB *vs.* hiLG+loAB	loLG+hiAB *vs.* loLG+loAB	hiLG+loAB *vs.* loLG+loAB
Number of DE-genes	432	407	489	441
Transcript Abundance >	311	111	129	293
Transcript Abundance <	121	296	360	148

Table 4. Top 10 canonical pathways for lean growth performance (a) and antibody titer (b) phenotype comparisons

Canonical Pathway	Pathway category	-log p-value	Genes involved	Transcript abundance	
				>	<
(a) Lean growth performance: high *vs.* low					
T Cell Receptor Signaling	1	3.84	13	12	1
PKCθ Signaling in T Lymphocytes	1	3.84	14	11	3
CD28 Signaling in T Helper Cells	1	3.84	14	10	4
CTLA4 Signaling in Cytotoxic T Lymphocytes	1	3.75	12	10	2
Role of NFAT in Regulation of the IR	1,2,3	3.42	16	11	5
Phospholipase C Signaling	3	3.42	19	13	6
iCOS-iCOSL Signaling in T Helper Cells	1	3.16	12	8	4
Protein Kinase A Signaling	3	3.16	25	14	11
Calcium-induced T Lymphocyte Apoptosis	4,1	2.99	9	7	2
CCR5 Signaling in Macrophages	1,5	2.63	9	7	2
(b) Antibody titer: high *vs.* low					
Integrin Signaling	3,6,7	8.08	24	1	23
Phospholipase C Signaling	3	4.86	21	4	17
Clathrin-mediated Endocytosis Signaling	1,8,9	4.70	18	1	17
IL-8 Signaling	1,5	4.70	18	3	15
Thrombin Signaling	10	4.56	18	2	16
Role of NFAT in Regulation of the IR	1,2,3	4.56	17	3	14
PTEN Signaling	4	4.56	14	0	14
P2Y Purigenic Receptor Signaling Pathway	10	4.26	14	2	12
Ephrin Receptor Signaling	9	4.01	16	2	14
CXCR4 Signaling	1,5	3.94	15	1	14

Pathway categories:
1 Cellular immune response; 2 Humoral immune response;
3 Intracellular and second messenger signalling; 4 Apoptosis;
5 Cytokine signalling; 6 Cellular growth, proliferation and development;
7 Cell cycle regulation; 8 Pathogen-influenced signaling;
9 Organismal growth and development; 10 Cardiovascular signaling;
Abbreviation: IR Immune response

Table 5. Top 15 biofunctions for lean growth performance (a) and antibody titer (b) phenotype comparisons

Annotated functions	Activation z-score	-log p-value	# Genes involved	Predicted activation state
(a) Lean growth performance: high *vs.* low				
organismal death	-4.39	2.90	77	<
development of leukocytes	3.51	4.29	39	>
differentiation of lymphocytes	3.38	6.46	40	>
cellular homeostasis	3.35	5.15	74	>
lymphocyte homeostasis	3.31	4.14	36	>
T cell development	3.28	4.04	34	>
development of blood cells	3.25	3.94	41	>
development of lymphocytes	3.23	4.18	37	>
T cell homeostasis	3.18	4.19	35	>
development of cardiovascular system	3.04	3.79	56	>
differentiation of mononuclear leucocytes	2.97	6.46	42	>
development of blood vessel	2.94	2.59	42	>
differentiation of B lymphocytes	2.78	3.48	19	>
differentiation of leucocytes	2.70	6.25	47	>
quantity of helper T lymphocytes	2.59	2.54	15	>
(b) Antibody titer: high *vs.* low				
adhesion of blood cells	-4.01	1.75	22	<
aggregation of cells	-3.92	3.68	24	<
aggregation of blood cells	-3.58	3.97	21	<
aggregation of blood platelets	-3.32	4.60	142	<
proliferation of cells	2.99	5.52	83	<
differentiation of cells	-2.96	3.03	25	<
cell transformation	-2.79	1.89	34	<
differentiation of blood cells	-2.78	1.49	43	<
activation of cells	-2.61	1.64	98	<
cell movement	-2.48	7.32	23	<
hemostasis	-2.47	5.77	39	<
activation of blood cells	-2.38	2.37	89	<
migration of cells	-2.37	6.50	34	<
proliferation of T lymphocytes	-2.36	2.52	55	<
cellular homeostasis	-2.31	1.56	35	<

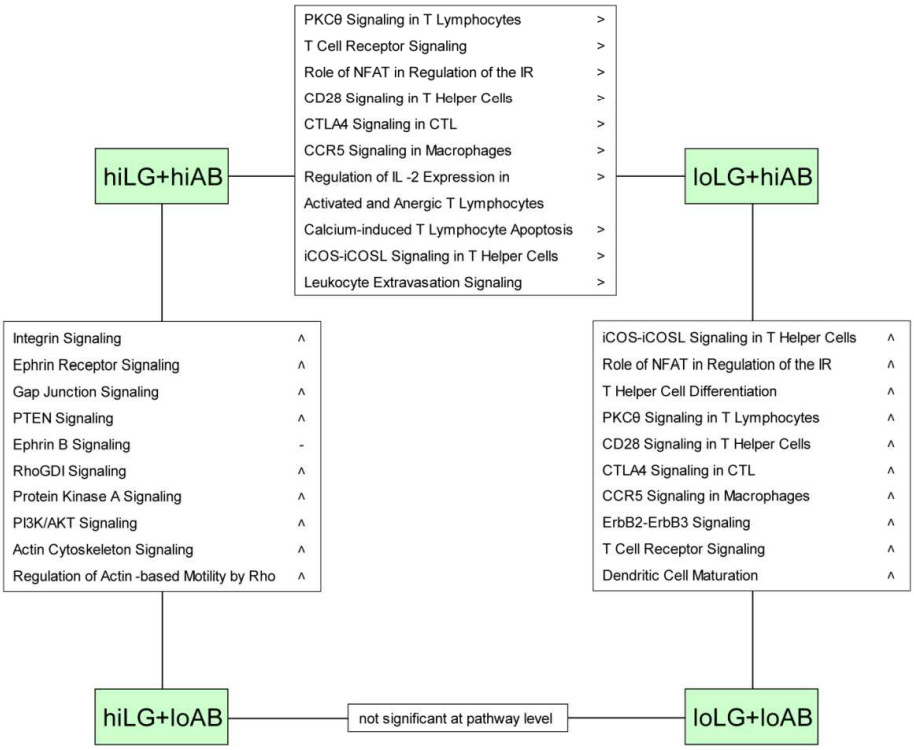

Figure 2. Affected canonical pathways revealed by differentiated comparisons of four phenotypic groups. Symbols > and < refer to overall transcript abundance and were set if more than 75 % of involved DE-genes showed positive or negative fold change, respectively.

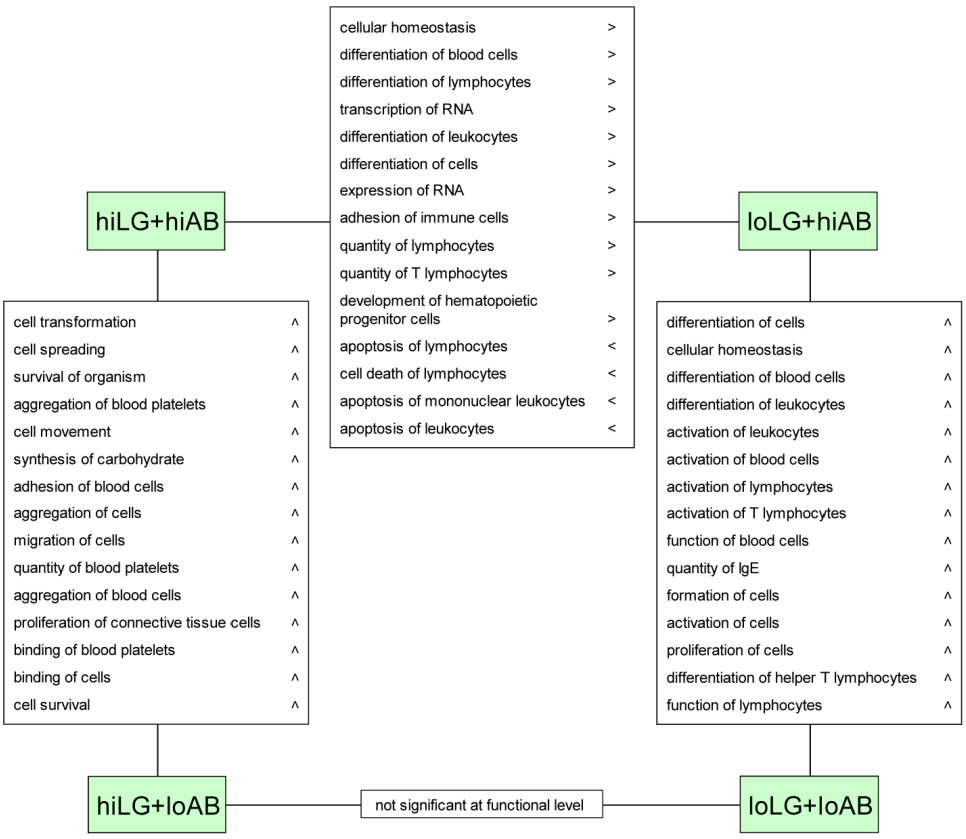

Figure 3. Altered biofunctions revealed by differentiated comparison between four phenotypic groups. Symbols > and < refer to IPA prediction of increased (>) or decreased (<) activation state of the respective biofunction.

Discussion

In animal farming, the relationship between performance traits and immunocompetence, its genetic foundation, and the consequences for animal breeding are debated but poorly understood. Previous work addressed the physiological costs of immune responses leading to disease resistance [25]. In addition, there are numerous reports about how certain factors like pathogen infection, poor hygiene conditions or physical and psychological stressors led to a metabolic impairment and thus to lower performance [25]. The resource allocation theory postulates the existence of metabolic constraints between immune function and growth performance [26,27]. However, immune activation and resulting metabolic impairments should be differentiated from genetically determined differences in immune responsiveness and performance potential in terms of weight gain, lean growth, and other production traits. Knap and Bishop [28] review evidence of genetic variation and discuss several breeding approaches to improve the genetic potential for immunocompetence.

Porcine selection for high immune response was found associated with enhanced weight gain [8,9], whereas selection for lean growth was observed associated with higher numbers of several leukocyte subtypes [10]. In addition, Galina-Pantoja *et al.* [29] provide evidence for the association of several leukocyte subsets with growth traits. However, inverse correlations between antibody titers after vaccination and weight gain [7] and between PBMC subsets and daily weight gain have also been reported [30,31]. These results suggest an incomplete understanding of the correlation between immunocompetence and performance traits.

In pigs undifferentiated by phenotype, we observed a broad transcriptomic response to TT that comprises changes to the abundance of immune response, cellular growth, proliferation, development, intracellular messenger, and second messenger signaling transcripts [16]; therefore, we asked whether phenotype differentiation would affect these responses. The results presented here reveal differentially-modulated transcriptomic responses of immune pathways and broad processes of cellular reorganization with respect to the phenotypic differences in lean growth performance and antibody response.

hiLG transcripts were increased for canonical pathways related to cellular immune response and biofunctions predicted to increase lymphocyte differentiation, development, and homeostasis, but to decrease leukocyte apoptosis, compared to loLG transcripts. These findings are concurrent with the more differentiated comparison of hiLG+hiAB *vs.* loLG+hiAB.

Although the comparison of hiLG+loAB *vs.* loLG+loAB revealed 441 DE-genes, similar to the other differentiated comparisons, no significant pathway or affected biofunction was identified. Hence, the background of low humoral immune responsiveness provided no clear functional differences for divergent lean growth.

Among the most significant canonical pathways, several were related to T-cell activities. For example, T cell receptor (TCR) signaling is initiated after major histocompatibility complex-associated antigens are recognized and bound by the TCR-CD3 complex. Transcript abundances of CD3 and the co-stimulatory receptor CD8 increased in hiLG and hiLG+hiAB compared to loLG groups. In TCR signaling, antigen binding to TCR leads to an initial sequence of tyrosinase activity followed by further downstream signaling, which leads to transcriptional activation of several genes including *IL-2*, a key-cytokine of Th1 response. Moreover, we found elevated transcript abundance of *c-FOS* encoding a component of the transcription factor AP1 that activates cytokine and other immune effector genes. Higher abundance of CD8 transcripts in hiLG may indicate higher numbers of cytotoxic T lymphocytes (CTLs) or a higher rate of differentiation into CTLs. In addition to TCR signaling, the following pathways are closely related: PKCθ signaling in T lymphocytes; CD28 signaling in T helper cells; CTLA4 signaling in cytotoxic T lymphocytes; and NFAT regulation of the immune response. These signal transduction mechanisms are initiated by antigen recognition by TCR-CD3 and co-stimulation of CD28 to activate and differentiate T cells, which involve cell survival, proliferation, and the production of IL-2. The enrichment of these pathway genes indicates the predominance of cell-mediated immune responses. Thus, the high lean growth phenotype in our study was characterized by several favourable performance traits and a transcriptomic response indicative for high cellular immunocompetence, partly combined with a strong humoral defense.

hiAB and loAB comparison revealed the hiAB group had a lower abundance of transcripts related to canonical cellular biology pathways (integrin and phospholipase C signaling) and immune responses (clathrin-mediated endocytosis and IL-8 signaling). Functional analysis demonstrated cell adhesion, aggregation, proliferation, and differentiation among the predominant processes. However, depending on backgrounds of hiLG and loLG, respectively, the differentiated comparisons show differently affected functions (Fig. 3). This indicates that affected pathways with primary immune functions did not necessarily reflect TT AB titers. Similar observations that immune assays may not reflect immunocompetence were reported [32]. However, it should be taken into account that the lymphocyte fraction of peripheral blood is composed of a smaller fraction of B lymphocytes compared to T cells, which may impair to some extent the detection of shifted transcript abundances in B cells. The comparison of loLG+hiAB *vs.* loLG+loAB presented pathways responsible for cell-mediated immune responses and biofunctions predominated by differentiation and activation of lymphocytes. Since loAB phenotypes showed exclusively higher transcript abundances for cellular immune responses, compensatory effects between humoral and cellular immune responses visible only for low performance phenotypes may be present.

Against the background of hiLG (hiLG+hiAB *vs.* hiLG+loAB), no transcript abundance alterations were found for primary immune pathways. However, cellular and organismal growth, proliferation and development (integrin, ephrin receptor, and gap junction signaling), and intracellular and second messenger signaling (integrin, rhoGDI signaling) pathways were affected. Decreased transcript abundances in hiLG+hiAB compared to hiLG+loAB had predicted biofunctions in cell transformation, aggregation, binding, and survival. Given that hiLG+hiAB phenotypes possessed both high AB titers and strong transcriptional immune responses, the increased T-cell activation transcripts were possibly offset by lower expression of several cell signaling and reorganization functions. It remains to be examined whether lower expression of other functions impair physiological or metabolic functions.

AB divergent phenotype comparisons revealed integrin signaling as the most significant pathway. Integrin DE-genes *ITGA2, ITGA2B,* and *ITGB3* increased 1.2- to 1.4-fold in loAB compared to hiAB; transcripts increased 1.4- to 1.6-fold in hiLG+loAB compared to hiLG+hiAB (Supplementary Table 2). Integrins are transmembrane molecules that enable attachment to and communication with other cells or the extracellular matrix. Although the IPA database does not attribute integrin signaling to the immune system, integrins may play a crucial role in the leukocyte immune response. Hogg and colleagues [33] showed that integrin-mediated leukocyte migration through the body and into lymph nodes is essential for immune responses. Moreover, integrins are in-

volved in the interaction between T cells and antigen-presenting cells by both synaptic adhesion and direct signaling [34].

Balanced immunocompetence without polarity of either cellular-mediated (Th1) or humoral (Th2) immune responses is a proposed selection goal to avoid resistance to specific infections/diseases or increased susceptibility to others [3]. Thus, a balanced Th1/Th2 phenotype, rather than extreme Th1 or Th2 responders, is desired for general disease resistance [35]. Because cytokine production and Th1/Th2 ratios are highly variable within a pig population [35-37], one can assume that the prevalence of either Th1 or Th2 responses is genetically fixed. Our observations of considerable transcriptomic differences between humoral and cellular-mediated immune responses corroborate these results. One might speculate that loAB individuals activate a higher cellular immune response to compensate for lower antibody-mediated immunity. Immune tolerance is an alternative host strategy to deal with pathogens [38] that is discussed for possible integration in selective pig breeding [39-41].

Recent research reported further insight into the relationship between obesity and adipose tissue inflammation [42]. Increased numbers of macrophages and T cells and their production of pro-inflammatory cytokines cause obesity-associated insulin resistance in several tissue types [43,44]. Association of adiposity with metabolic impairments and a low-grade inflammatory state was also shown in pig [45]. Assuming a higher susceptibility for insulin resistance in obese animals from our experimental population, the immune status of low performance animals may differ from that of lean growth animals. Further, if adipose tissue can contribute to disease resistance or host tolerance through the systemic immune system, the relationship between fat and immunocompetence becomes more complex. These factors will have to be considered for the integration of genetics and phenotype expression into breeding programs.

Conclusions

We observed significant functional transcript abundance changes during the immune response to TT for both divergent lean growth performance and humoral immune responses. High lean growth showed activated cellular immune responses related to lymphocyte differentiation, development, and homeostasis compared to low lean growth phenotypes. Increased transcript abundances for immune activation were possibly offset by decreased expression of several cell signaling and reorganization functions; however, numerous favourable performance traits suggest a lack of physiological or metabolic impairment. The majority of affected primary immune pathways did not reflect AB titer differences. Particularly, in low lean growth performers low humoral immune responders revealed higher transcriptional activation of cellular immune responses than low AB responders, suggesting compensatory effects between humoral and cellular immune responses. However, the group of the most desired animals with high lean growth and high humoral immune response (hiLG+hiAB) revealed also higher transcriptional activation of cellular immune responses compared to the low performing group. Though the above-mentioned compensatory effects may exist, this finding demonstrates the occurrence of individuals with superior performance combined with high Th1 and Th2 immune responsiveness.

Supplemental Material

Supplementary Table 1: Loads of factor 1.
http://www.ijbs.com/v09p0907s1.pdf
Supplementary Table 2: DE-genes of all comparisons.
http://www.ijbs.com/v09p0907s2.xls

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Competing Interests

The authors have declared that no competing interest exists.

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2.4 PBMC transcriptomic responses to primary and secondary vaccination differ due to divergent lean growth and antibody titers in a pig model

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Content summary:

PBMC responses to immune stimulation were analysed within different phenotypes for the primary response from day 0 to day 14 and for the adaptive response as comparison between day 14 and day 28. The primary response at day 14 revealed a general decrease of transcript frequencies for various signaling pathways. The adaptive response, however, was characterized by increased activation of immune responses in the high lean growth (LG) phenotype. Low LG animals, however, showed only weak responses concerning transcript abundance changes.

Author contributions:

M. Adler (100 %) conceived and investigated the differences of responses to primary and secondary vaccination for differentiated phenotypes. M. Adler (50 %) and K. Wimmers performed vaccination, blood sampling and PBMC preparation. M. Adler (100 %) performed the Anti-TT antibody ELISA assays. M. Adler (80 %) and K. Wimmers collected phenotype data of productive traits and performed the principal component analysis. M. Adler (100 %) performed RNA isolation and prepared the microarray analyses. M. Adler (90 %) and K. Wimmers analysed the microarray data. M. Adler (90 %) and K. Wimmers wrote the paper.

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PBMC transcriptomic responses to primary and secondary vaccination differ due to divergent lean growth and antibody titers in a pig model

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Adler M, Murani E, Ponsuksili S, Wimmers K. PBMC transcriptomic responses to primary and secondary vaccination differ due to divergent lean growth and antibody titers in a pig model. *Physiol Genomics* 47: 470–478, 2015. First published July 14, 2015; doi:10.1152/physiolgenomics.00015.2015.—The genetic relationship between immune responsiveness and performance is not well understood, but a major topic of the evolution of resource allocation and of relevance in human medicine and livestock breeding, for instance. This study aims to show differences of transcript abundance changes during the time intervals before and after two tetanus toxoid (TT) vaccinations in domestic pigs differing in lean growth (LG) and anti-TT-antibody titers (AB) parameters of performance and immunocompetence. During response to the first vaccination all animals had a general decrease in transcript abundances related to various functional pathways as measured by comparative Affymetrix microarray hybridization and Ingenuity Pathway analyses. Low-AB phenotypes had predominantly decreased immune response transcripts. Combined phenotypes high-AB/high-LG had decreased transcripts related to growth factor signaling pathways. However, during the interval after the booster vaccination, high-LG and high-AB animals responded exclusively with increased immune transcripts, such as B-cell receptor signaling and cellular immune response pathways. In addition, high-LG and low-AB animals had predominantly increased transcripts of several cellular immune response pathways. Conversely, low-LG and high-AB animals had few elevated immune transcripts and decreased transcripts related to only two nonimmune-specific pathways. In response to booster vaccination high-LG phenotypes revealed enriched transcripts related to several different immune response pathways, regardless of AB phenotype. Different from the expected impact of AB titers, divergent AB phenotypes did not reflect considerable differences between immune transcripts. However, highly significant differences observed among divergent LG phenotypes suggest the compatibility of high performance and high vaccine responses.

leukocytes; pathway analysis; immune response; performance; microarray;

INTER- AND INTRABREED VARIATION of immunocompetence in farm animals is of high interest for resource-efficient animal production at high animal health and welfare conditions. However, strong selection for production traits is suspected to lead to impaired immune function (36, 39), thus there is all the more need for comprehensive selection approaches that include traits for disease resistance and immunocompetence (24, 27, 40, 42, 46).

Because genetic variation to immune stimuli is reported in pigs (11–13, 18, 44), selection for high immune response phenotypes should be feasible. However, the direct relationship between immune responsiveness and performance traits, which are of major interest in breeding programs, is not well understood and implications from various studies are not clear-cut regarding the value and the direction of the relationship between immunity and performance. For example, it was reported that weight gain negatively correlates with antibody titers against *Bordetella bronchiseptica* and pseudorabies in crossbred pigs (32). Similarly, average daily weight gain negatively correlates with quantities of several lymphocyte subsets, although proportions of SLA-DQ-positive cells positively correlate with carcass weight and feed conversion (20). In addition, several studies demonstrate that peripheral blood mononuclear cell (PBMC) subsets are heritable and negatively correlate, phenotypically and genetically, with daily gain performance (7, 8). Conversely, selection for high humoral and cellular immune responses in Yorkshire pigs is associated with enhanced weight gain (31, 46, 47), but these high immune response animals are also prone to develop more severe arthritis (30, 31). In Large White pigs selected for high or low lean growth (LG) under restricted feeding, high LG animals have higher quantities of several types of lymphocytes and monocytes, although no associations are observed between divergent selection lines of food intake and LG levels under ad libitum feeding (6). To enable consequent genetic improvement of performance and immune traits, knowledge of the functional links between metabolic and immune (signaling) pathways is required in addition to phenotypic and genetic trait correlations. Insights into the functional networks synergistic, antagonistic, or independently affecting performance and immune responsiveness will also have implications for medicine, in particular sports medicine, and facilitate the drug development toward supporting immune defense in mentally or physically stressful living conditions.

Recently, studies of transcriptional responses of single genes have been complemented by transcriptomic techniques, which provide a powerful tool to examine genome-wide alterations of gene regulation after pathogen infection (4, 23, 43) or immune stimulation (2, 21, 48). Identification of key genes and associated molecular pathways allows better understanding of immune responses. Furthermore, the pig provides an excellent animal model in biomedical genomics (29) because understanding the porcine immune system can provide insight into human infectious diseases and host responses (14, 16, 17, 22).

In a previous study of genome-wide effects of immune stimulation by vaccination against tetanus toxoid (TT) in PBMCs, we observed in vivo a broad transcriptomic response, which comprises changes to the abundance of immune response, cellular growth, proliferation, development, intracellular messenger, and second messenger signaling transcripts (2, 35). TT vaccination induces an extensive immune response involving both the cellular (Th1) and humoral (Th2) branches of the immune system (15, 28, 41). TT represents a nonubiquitous antigen in swine for which weaning piglets are consid-

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ered antigen-naïve and therefore provides a suitable model antigen for immune stimulation. Using this model antigen we have previously analyzed the transcript abundance in PBMCs depending on lean growth (LG) performance and anti-TT antibody (AB) titers (36). However, this analysis does not allow any information about temporal dynamics of transcript abundances during the response to two vaccination events. In fact, major changes of transcript abundance due to primary and secondary vaccination can be expected that are obligatory and underlie only subtle biological variation. The suspected interrelation of performance and immune traits can be addressed as the differences of the vaccination-induced changes of transcript abundance between well-defined groups of probands of divergent combinations. Here, to reveal more insights into the time course of transcript abundance changes, we made these comparisons in the intervals from pre- to postvaccination among animals divergent for LG and AB titers (Fig. 1). Our interest was to identify and characterize the time interval that shows clear differences between phenotypes during the switch from innate to adaptive immune response. Based on significantly different transcript abundances, canonical signaling pathways and biofunctions were addressed to four groups of combined phenotypes of divergent performance and humoral immune response.

MATERIALS AND METHODS

Animals and vaccination. Animal care, vaccination, and blood collection were performed according to the guidelines of the German Law of Animal Protection. The experimental protocol was approved by the Animal Care Committee of the Leibniz Institute of Farm Animal Biology and the State Mecklenburg-Vorpommern (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei; LALLF M-V/TSD/7221.3-2.1-020/09).

The experimental design is outlined in Fig. 1. Animals, vaccination, and blood sampling were described previously (3). In brief, a total of 160 5 wk old male and female piglets of a German Landrace outbred herd with known pedigree were initially vaccinated (*day 0*), and a booster vaccination was given at *day 14*. Vaccination was performed by 1 ml (30 IU) of TT vaccine (Equilis Tetanus-Vaccine, Intervet, Unterschleißheim, Germany) composed of TT and aluminum hydroxide as adjuvant. Of each animal 6 ml of venous blood was taken at the vena cava craniales into EDTA-coated tubes at *days 0, 14*, and *28*. Juvenile animals (average age of 10 wk) were performance-tested during fattening and at termination (final average weight of 110 kg) according to the guidelines of the German performance test (50). Resulting from a principal component analysis of 32 traits related to performance, growth, body composition, and meat properties, the first factor was taken as a basis for the identification of LG performance phenotypes (3). Most significant parameters for LG were lean meat content, loin eye area, meat-to-fat ratio, and (negatively signed) fat area and backfat. Animals from the respective terciles of highest and lowest factor one values were categorized as high (hiLG) and low (loLG) LG performance, respectively.

Blood and plasma samples. Blood sampling was performed between 8:00 and 9:00 AM. EDTA blood samples were collected on ice until PBMC preparation. Plasma samples obtained during PBMC isolation were stored at −80°C until further analysis. Time until freezing at −80°C was about 30 min for plasma and 90 min for PBMC.

Plasma AB titers (all isotypes of anti-TT) at *day 14* and *day 28* were determined in triplicate using a commercially available ELISA

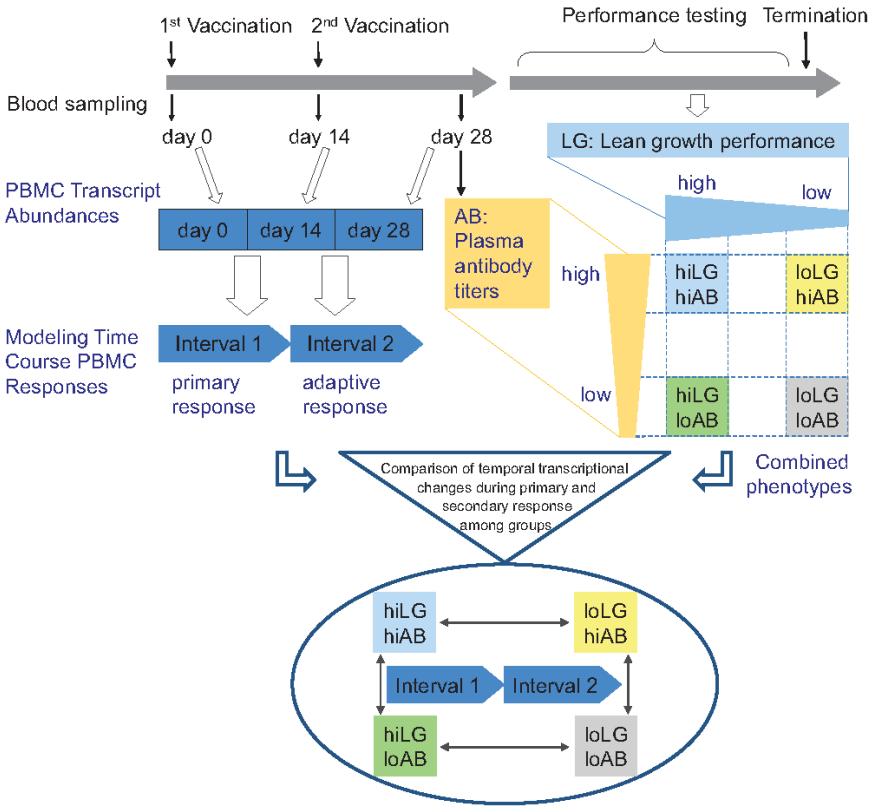

Fig. 1. Experimental design. Five-week-old piglets were vaccinated twice with tetanus toxoid (TT). Directly before the initial (*day 0*) and booster (*day 14*) vaccination and at *day 28*, peripheral blood mononuclear cells (PBMCs) were isolated from collected blood samples. ELISA quantified anti-TT antibody (AB) titers from *day 28* plasma samples. Animals were performance-tested, and performance data and AB titers provided a basis for identification of divergent phenotypes of lean growth (LG) performance and humoral immune response, respectively. Expression profiles resulting from both the transition from *day 0* to *14* and from *day 14* to *day 28* were analyzed and compared between the 4 phenotypes by microarray analyses.

(RE57441; IBL International, Hamburg, Germany) according to manufacturer's directions. According to LG phenotype ratings, animals assigned to the first and third terciles of *day 28* AB titers were rated as high (hiAB) and low (loAB) humoral immune response phenotypes, respectively. By combination of LG and AB phenotypes four groups of differentiated phenotypes were set up: hiLG+hiAB, hiLG+loAB, loLG+hiAB, and loLG+loAB. In turn, from each of these groups 10 animals were randomly selected for microarray analyses.

RNA preparation and microarray hybridization. PBMC isolation, RNA isolation, and target preparation were performed as described (3). In brief, PBMCs were isolated from 6 ml blood samples by centrifugation on Histopaque density gradients (Sigma-Aldrich, Taufkirchen, Germany). Total RNA was isolated using Qiazol reagent (Qiagen, Hilden, Germany), treated with DNase, and column-purified using the RNeasy Mini Kit (Qiagen). Absence of DNA contamination was assessed by PCR amplification of porcine *GAPDH* (forward primer: 5′-AAGCAGGGATGATGTTCTGG-3′; reverse primer: 5′-ATGCCTCCTGTACCACCAAC-3′). Each RNA sample was transcribed to DNA using the Ambion WT Expression Kit (Ambion, Austin, TX). DNA preparations were fragmented and labeled with a WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA). Labeled cDNA was hybridized on Affymetrix snowball arrays (16, 19). Microarray data are MIAME-compliant and were deposited in the Gene Expression Omnibus (GEO) repository (see below).

Data processing and functional analyses. The dataset supporting the results of this article is available in the GEO repository of the National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/geo (GEO: GSE47845).

To normalize quality-controlled raw data, the PLIER algorithm was applied using Expression Console 1.1 software (Affymetrix). Expression data were filtered by standard deviation ($s \leq 0.2$). Changes of relative transcript abundance were determined by mixed model analysis, which is a well-established approach for dissection of variant data (34, 37). The model includes effects of sire, AB phenotype (hi or lo), LG phenotype (hi or lo), time (*day 0, 14*, or *28*), and interactions between AB, LG, and time. Accordingly, the model [$y = \mu + S + T + AB + LG + (AB \times LG) + (T \times AB \times LG) + \varepsilon$] was fitted using the JMP Genomics 5.0 software (SAS Institute, Cary, NC). The model was combined with a repeated statement for the time component to take into account correlations among measurements made on the same subject by specifying a heterogeneous covariance structure. The three-way interaction between LG phenotype, AB phenotype, and time refers to the longitudinal experimental design and represents the 12 experimental units [2 (LG) × 2 (AB) × 3 (T)] that were defined. Comparisons of (*day 0* vs. *day 14*) and (*day 14* vs. *day 28*) were made within each of four groups of differentiated phenotypes hiLG+hiAB, hiLG+loAB, loLG+hiAB, and loLG+loAB based on the least square means of the three-way interaction. Subsequently, we compared the eight lists (2 comparisons × 4 phenotype groups) of transcripts with significant different abundance to discriminate temporal changes of transcription that are common among phenotype groups and that are unique for a particular phenotype group (Fig. 2). Annotation data for the snowball arrays were obtained from the developers (19). Significantly altered transcripts ($P < 0.05$) were assigned to annotated genes and bioinformatically analyzed by Ingenuity Pathways Analysis (IPA) software (49). Affected canonical pathways and downstream biofunctions were subjected to IPA's Benjamini-Hochberg multiple testing correction P value procedure (5). Cut-off criteria were set for canonical pathways to false discovery rate (FDR)-corrected P values < 0.05; for predictions of altered biofunctions, cut-off values were FDR-corrected P values < 0.05 and absolute values of activation z-scores > 2.0. We used IPA's compare data tool to create Venn diagrams and calculate unique and common differentially expressed (DE) genes among phenotypes.

RESULTS

Blood samples for comparative microarray analyses and determination of AB titers were taken at *day 0* (prevaccination) and *14* (before booster vaccination) and at *day 28*, i.e., 14 days after second vaccination. (Fig. 1). AB titers at *day 14* were generally below the lower assay detection limit (<0.08 IU/ml). AB titers at *day 28* ranged from <0.1 IU/ml to >1.0 IU/ml (mean = 0.33 IU/ml; standard deviation = 0.23 IU/ml) and were used as a basis for the identification of divergent phenotypes of high humoral immune response (hiAB, mean = 0.57 IU/ml, standard deviation = 0.13 IU/ml) and low humoral immune response (loAB, mean = 0.23 IU/mL, standard deviation = 0.04 IU/ml; $P < 0.001$).

Within each phenotype group, hiLG+hiAB, hiLG+loAB, loLG+hiAB, or loLG+loABl comparisons for the intervals from *day 0* to *day 14* (primary immune response, *interval 1*) and *day 14* to *day 28* (secondary immune response, *interval 2*) revealed changes of abundance of 433 to 1,770 transcripts; the numbers of genes with significantly different transcript abundances from each group and overlapping gene numbers among comparisons are illustrated by Venn diagrams in Fig. 2. The common and phenotype group-specific DE-specific temporal changes of transcript abundances among groups were considered in subsequent IPAs (49).

IPA genes with significantly different transcript abundances (hereafter referred to as DE genes) were assigned to canonical pathways (Fig. 3) and biofunctions, as defined in the Ingenuity Knowledge Base (Supplemental Table S1).[1] Comparisons between *day 0* and *day 14* identified between 571 and 1,770 DE genes and revealed different proportions between increased and decreased transcript abundances among the four phenotypes at *day 14* (Table 1). In response to the first vaccination for all groups significant canonical pathways presented generally decreased transcript abundances (Fig. 3). Accordingly, all affected biofunctions were predicted for decreased activation (Supplemental Table S1).

Response of high LG phenotypes to first vaccination. Comparison of hiLG animals between *day 0* and *day 14* revealed 1,201 and 1,770 DE genes for high and low AB responders, respectively (Table 1). DE genes with decreased transcript abundances from the hiLG+hiAB group were related to canonical pathways of several growth factors (ErbB signaling, GDNF family ligand-receptor interactions, PDGF signaling) and cellular immune response (LPS-stimulated MAPK signaling, IL-6 signaling, IL-3 signaling) (Fig. 3). Additional pathway analyses of DE genes unique (see Venn diagrams in Fig. 2) to hiLG+hiAB in contrast to hiLG+loAB and loLG+hiAB phenotypes, respectively, revealed that certain pathways are exclusively affected in the hiLG+hiAB group (ErbB Signaling, GDNF family ligand-receptor interactions, LPS-stimulated MAPK signaling; Supplemental Table S2). The most significant biofunctions predicted to decrease were related to cellular proliferation, leukocyte function, inflammatory response, and apoptosis of antigen-presenting cells (Supplemental Table S1).

The hiLG+loAB group had decreased transcripts related to canonical pathways predominated by cellular immune re-

[1] The online version of this article contains supplemental material.

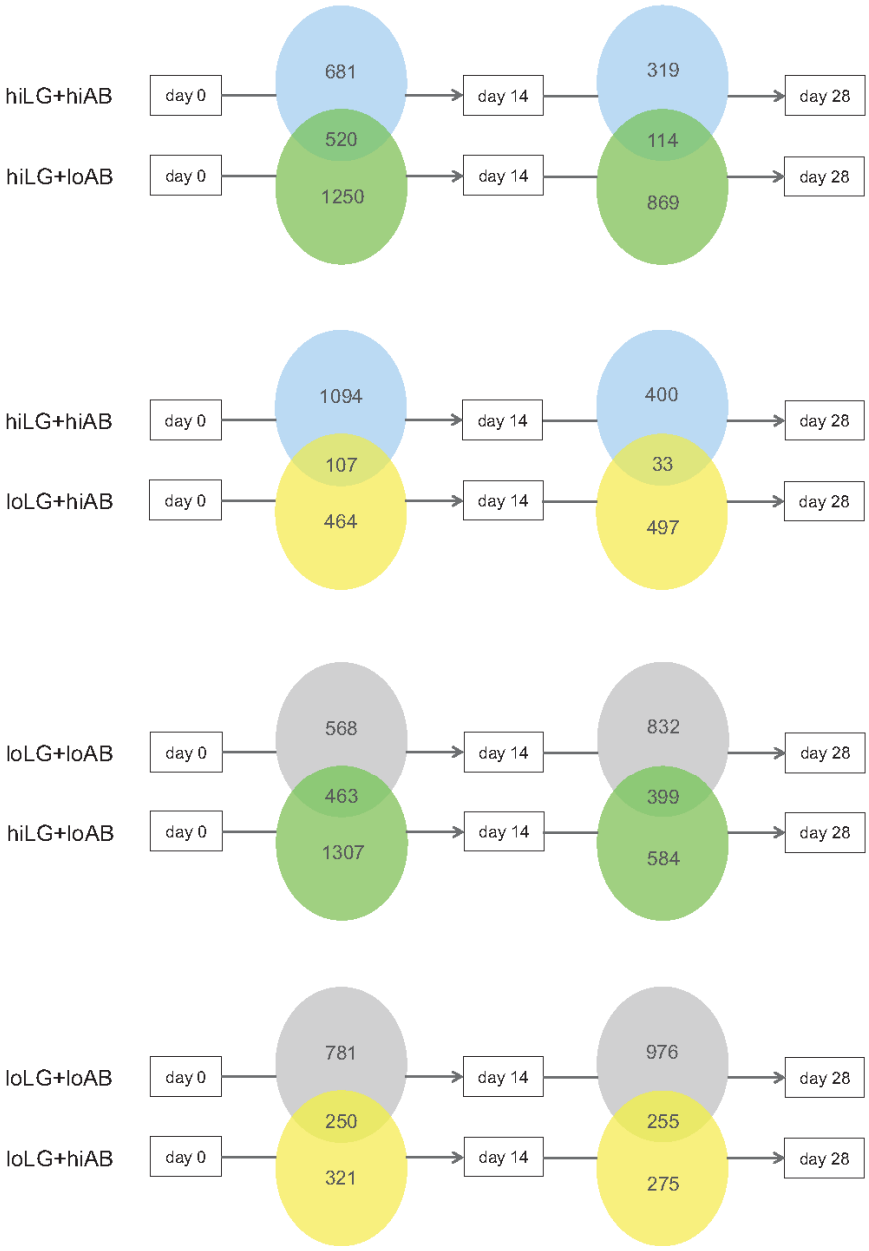

Fig. 2. Venn diagrams of unique and common differentially expressed (DE) genes in *intervals 1* and *2* among the 4 phenotypes. Comparative microarray analyses revealed different transcript abundances (DE genes), as indicated by numbers, among groups between *day 0* and *day 14* (*interval 1*) and *day 14* and *day 28* (*interval 2*), respectively.

sponses, protein ubiquitination, and cellular or oxidative stress (NRF2-mediated oxidative stress response, mitochondrial dysfunction) (Fig. 3). Moreover, protein ubiquitination, mitochondrial dysfunction, and the NFAT pathway were characterized as unique for this group (see Supplemental Table S2). Affected biofunctions were predicted to decrease quantities of blood cells, particularly mononuclear leukocytes and hematopoietic cells, and hematopoietic progenitor cells (Supplemental Table S1).

Response of low LG phenotypes to first vaccination. The loLG+hiAB group had fewer significantly different transcript abundances with only 572 DE genes for *interval 1*, whereas the loLG+loAB group had 1,032 DE genes (Table 1). Accordingly, those transcripts were only related to pathways of protein ubiquitination and CTLA4 signaling in cytotoxic T lymphocytes (Fig. 3). Affected biofunctions were predicted to decrease quantity of blood cells and expansion of leukocytes (Supplemental Table S1).

The loLG+loAB group presented DE genes with mainly immune response-related pathways, similar to hiLG+loAB animals. Although these two groups shared five affected pathways, examination revealed unique DE genes constitute these pathways (CD28 signaling in T helper cells, calcium-induced T lymphocyte apoptosis, and NF-κB signaling) (Fig. 3). Altered

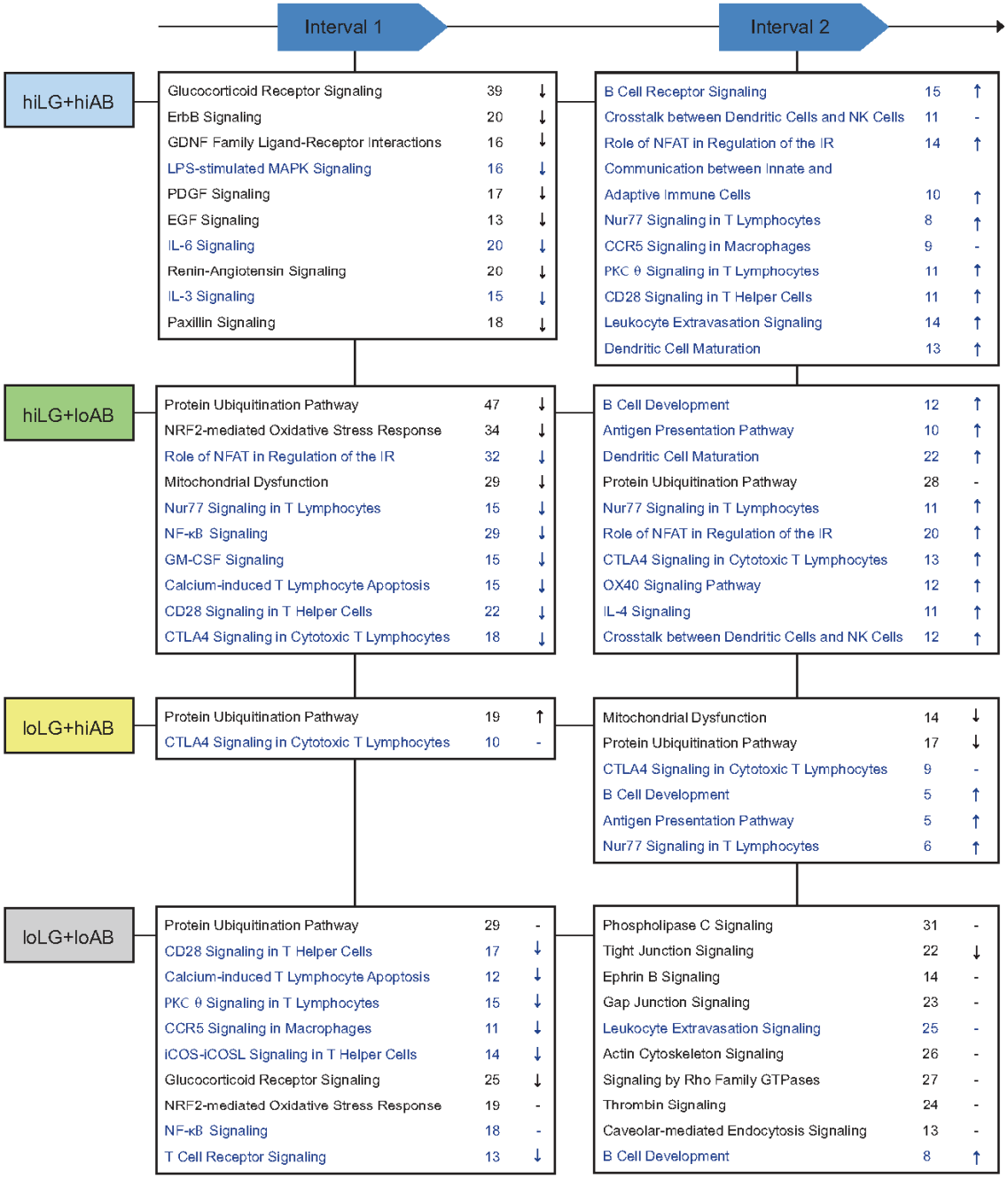

Fig. 3. Affected canonical pathways revealed by comparisons of the 4 pig phenotypes. Ingenuity Pathway analyses identified canonical pathways related to transcript abundances of differentially expressed genes (DE genes) from comparative microarray analysis of each pig phenotype after initial TT vaccination (primary response) or booster vaccination (secondary response). For each phenotype, affected pathways and the number of DE genes assigned to that pathway are given. Symbols indicate increased (↑), decreased (↓), or unchanged (-) transcript abundance and were given if >75% of involved DE genes showed positive or negative fold change, respectively. Pathways with primary immune function are shown in blue.

biofunctions were predicted to decrease quantity of leukocytes including hematopoietic stem cells and to decrease expression or transcription of RNA.

Response of high LG phenotypes to second vaccination. Comparison of hiLG animals between *day 14* and *day 28* revealed 442 and 994 DE genes for high and low AB responders, respectively (Table 1). Significant canonical pathways generally exhibit elevated transcript abundances. Accordingly, affected biofunctions were predicted for increased activation.

Table 1. *Numbers of DE genes in the intervals between days 0 and day 14 as well as day 14 and day 28, i.e., primary and secondary response to vaccination, for divergent phenotypes of lean growth performance and antibody titers*

	Differentiated Phenotypes of High and Low LG and AB			
	hiLG+hiAB	hiLG+loAB	loLG+hiAB	loLG+loAB
Interval 1: day 0–day 14				
DE genes, *n*	1,201	1,770	571	1,031
Transcript abundance ↑	292 (24%)	491 (28%)	281 (49%)	360 (35%)
Transcript abundance ↓	909 (76%)	1279 (72%)	290 (51%)	671 (65%)
Interval 2: day 14–day 28				
DE genes, *n*	433	983	530	1,231
Transcript abundance ↑	292 (67%)	519 (53%)	180 (34%)	607 (49%)
Transcript abundance ↓	141 (33%)	464 (47%)	350 (66%)	624 (51%)

DE, differentially expressed; LG, lean growth; AB, antibody titers; hi, high; lo, low.

For the hiLG+hiAB group, transcripts were predominantly related to canonical pathways of humoral and cellular immune responses, particularly B cell receptor (BCR) signaling (Fig. 3). BCR signaling is the crucial pathway for B cell activation in the humoral immune response and comprises antigen recognition by BCRs and intracellular signal transduction to B cell proliferation, AB production and secretion, memory B cell differentiation, cell survival, and apoptosis (10). Enrichment of BCR signaling-related transcripts was found uniquely in the hiLG+hiAB phenotype (see Supplemental Table S2). Other significant pathways were related to T-cell-mediated immune response, including NFAT regulation of immune response, Nur77 signaling, and PKCθ signaling in T lymphocytes. The most significant predicted biofunctions were increased cell proliferation, particularly of lymphocytes (Supplemental Table S1).

The hiLG+loAB group also showed a predominance of unique signaling pathways related to the immune response, such as B cell development, antigen presentation, and dendritic cell maturation (Fig. 3). Significant predicted biofunctions at *day 28* were related to increased quantity, differentiation, and development of leukocytes (Supplemental Table S1).

Response of low LG phenotypes to second vaccination. In response to the booster vaccination given at *day 14*, loLG+hiAB animals presented 533 DE genes and loLG+loAB animals presented 1,239 DE genes (Table 1). For the loLG+hiAB group, IPAs revealed decreased transcripts related to mitochondrial dysfunction and protein ubiquitination pathways and increased transcripts related to immune response signaling (B cell development, antigen presentation, and Nur77 signaling in T lymphocytes) (Fig. 3). However, no corresponding biofunctions were identified (Supplemental Table S1). Additional pathway analyses of unique genes (see Venn diagrams in Fig. 2 and Supplemental Table S2) in contrast to loLG+loAB or hiLG+hiAB failed to clearly characterize this phenotype. B cell development and Nur77 signaling appeared as unique by comparison to loLG+loAB, and mitochondrial dysfunction, protein ubiquitination, and antigen presentation were found among unique DE genes in contrast to hiLG+hiAB.

The loLG+loAB group showed a balance between increased and decreased transcript abundances within significant pathways related to intracellular and second messenger signaling, cellular and organismal growth and development, and cellular immune responses (Fig. 3). Only tight junction signaling transcripts decreased, while only B cell development transcripts increased. Pathway analyses of unique DE genes revealed phospholipase C, tight junction, gap junction, and ephrin B signaling as affected only in the loLG+loAB phenotype. Affected biofunctions were related to increased quantity of mononuclear leukocytes or B lymphocytes and hematopoietic progenitor cells and to decreased activation, aggregation, and engulfment of blood cells (Supplemental Table S1). The observed clear contrasts between the high and low LG groups in terms of canonical pathways were poorly reflected by proportions of shared and unique DE genes, respectively (Fig. 2). While hiLG+hiAB compared with loLG+hiAB revealed only few shared DE genes (~ 8%) the comparison between hiLG+loAB and loLG+loAB showed a proportion of about 50% shared DE genes.

DISCUSSION

The present study explores time course effects of transcriptional responses and response differences between divergent LG and AB phenotypes. Comparison of *day 0* transcriptome profiles were performed among all groups but did not reveal significant differences in terms of affected canonical pathways or biofunctions (data not shown). This indicates that the phenotype groups did not differ before vaccination and the animals were in a naïve state before the antigenic challenge. It is to be assumed that the first vaccination induces an innate immune reaction, whereas the booster induces the adaptive immune system. AB titers at *day 14* were consistently low, approximately at the assay detection limit. It was not until the second vaccination that considerable AB titers were detected and thus high and low responders could be identified. Comparing *day 0* and *day 14*, all four phenotypes had a general decrease in transcripts of signaling pathways and predicted biofunctions. Significant pathways in loAB phenotypes were predominantly related to immune response, whereas hiAB and hiLG groups correlated with several growth factor signaling processes. No influence of hiLG or loLG phenotypes was observed. Decreased transcripts within significant pathways at *day 14* correlates with our previous study of multiple time points after an initial and booster vaccination (2). Within 24 h after the initial vaccination, transcripts related to immune responses and other biofunctions increased. However, at *day 14* both studies show more decreased transcripts, possibly suggesting tissue reorganization and cell population modification occur 2 wk after initial challenge.

After the second immune stimulation (i.e., *day 28*), hiLG animals respond exclusively with elevated transcripts of hu-

moral and cellular immune processes. Since BCR signaling is significant only in hiAB animals, the humoral response seems to be more pronounced in hiAB animals, whereas loAB animals may have a bias to the cellular branch.

The loLG phenotype revealed few significant pathways and biofunctions in the context of hiAB. A small number of gene transcripts assigned to immune functions were elevated, and two nonimmune-specific transcript pathways were decreased at *day 28*. LoLG+loAB animals had transcripts correlated with pathways assigned to multiple functions, but directionality could not be predicted because gene transcripts were not consistently increased or decreased.

Only hiLG phenotypes showed considerable activation of immune responses that can be expected after immune stimulation in the transition from *day 14* to *28*. Elevated cellular immune response transcripts were found in hiLG animals regardless of AB titers; however, humoral immune response-crucial BCR signaling was significant only in the hiAB group. BCR signaling is crucial for activation of B cells and comprises BCR antigen recognition (membrane-bound immunoglobulins and associated molecules CD79a and CD79b), signal transduction, and processing leading to gene transcription and altered cell metabolism and cytoskeletal organization (10). For the hiLG+hiAB phenotype, we found increased transcript abundances for the adapter proteins GAB, BLNK, and BAM32, which are involved in initial signal generation. In addition, Btk, p38, and JNK1/2 kinases and Oct-2, a B cell-specific transcription factor (26), transcripts were elevated. p38 and JNK1/2 enter the nucleus and activate several transcription factors. Although these results indicate the activation of B cell-mediated immune responses, it should be taken into account that the lymphocyte fraction of peripheral blood is composed of a smaller fraction of B lymphocytes than T cells, and moreover, within the T helper population, Th2 lymphocytes are less abundant than Th1 cells. Both may impair, to some extent, the detection of shifted transcript quantities in B cells and T helper cells indicative for the humoral immune response.

In summary, consistent with our previous studies (2, 3), hiLG phenotypes have enriched transcripts of immune response pathways in the context of both hiAB and loAB. In addition, our results reveal that only after booster vaccination can ongoing immune response activation be observed at the transcriptional level. To our surprise, AB phenotype differences did not considerably impact immune response pathways. Thus, under certain circumstances results from immune assays may not reflect the actual immunocompetence as previously reported (1).

However, differences between hiLG and loLG phenotypes became clearly visible in response to booster vaccination, as hiLG animals had numerous enriched immune response transcripts and loLG animals had few. Studies on the relationship between production performance and immune response have revealed similar results. Yorkshire pigs selected for high cellular and humoral immune responses also show increased weight gain (31, 46, 47). In particular, the authors state that "the reason for advantages in growth rate are not known but may reflect efficient response to clinical and subclinical infection with reduced duration of illness and reduced muscle growth" (46).

In general, it seems plausible to assume that animals with higher and therefore effective immune responses benefit from decreased incidence of infection and therefore unimpaired growth performance. A further explanation may be given by the fact that in pigs, exposure to stressors promotes catabolic processes. Stressed pigs therefore show reduced growth and decreased lean-to-fat ratios (25, 45). Hence, in addition to immune activation, the correlating stress induction may play a crucial role in metabolic effects. It is conceivable that hiLG animals either possess highly effective immune responses or are better able to handle the stressors caused by vaccination.

Further understanding of the interaction between the immune system and the metabolic processes important for the expression of production traits is needed. Several studies report that certain factors, such as infection, poor hygiene conditions, or physical and psychological stressors, lead to metabolic impairment and, thus, lower production performance. Furthermore, immune responses require physiological costs (9), and resource allocation between immune function and growth performance can be assumed (38). However, such immune system activation and resulting adverse effects on metabolism should be differentiated from genetically determined differences in immunocompetence and performance potential in terms of weight gain, LG, and other production traits. As already indicated, immune traits are heritable, and considerable individual variation of immune responses can be observed. Today's breeding programs are faced with demanding requirements (33), and the aim of integration of immune traits leading to disease resistance reconcilable with animal welfare underlines the necessity of a comprehensive understanding of the genetic control between them.

In conclusion, in response to booster vaccination, hiAB animals did not have pronounced activation of immune responses at the transcriptional level compared with loAB animals. However, LG phenotype animals revealed predominantly enriched immune response transcripts. For hiAB animals, BCR signaling was the most significant function, whereas loAB animals had enriched gene transcripts assigned mainly to cellular immunity. Hence, both hiLG phenotypes were characterized by several favorable production traits and a combined humoral and cellular immune response in hiAB animals, as well as a predominance of cellular immune response in loAB animals. The highly significant differences observed among divergent LG phenotypes on the one hand suggest the compatibility of LG and immunocompetence and, on the other hand, point to an important role of LG traits within the genetic interaction of metabolism and immunocompetence.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.A., S.P., and K.W. performed experiments; M.A., S.P., and K.W. analyzed data; M.A., E.M., S.P., and K.W. interpreted results of experiments; M.A. prepared figures; M.A. and K.W. drafted manuscript; M.A., E.M., S.P., and K.W. edited and revised manuscript; M.A., E.M., S.P., and K.W. approved final version of manuscript; E.M., S.P., and K.W. conception and design of research.

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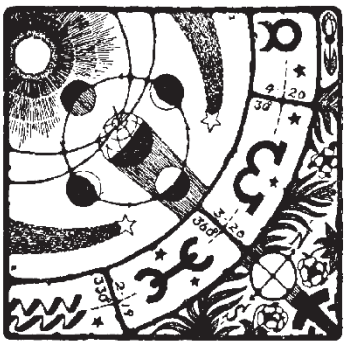

3 Discussion

The studies underlying this thesis evaluated the analysis of genome wides responses of porcine PBMCs to experimental TT vaccination and then compared and dissected longitudinal expression profiles from divergent phenotypes differentiated for high and low lean growth performance and high and low anti-TT titers, respectively.
Significant differences of relative mRNA abundances between time stages or phenotypes enabled the identification of differentially expressed genes which are involved in affected canonical pathways of molecular signaling processes.
The general discussion of this thesis will attempt to evaluate assets and drawbacks of microarray based analysis of PBMC transcript frequencies, to provide an overall characterization of the transcriptional responses observed in the pilot study and in the ongoing dissections of differentiated phenotypes and finally, to infer implications for both important traits in pig biology: leaness and immunocompetence.

3.1 PBMC Transcriptome Analyses by Microarrays: Advantages and Drawbacks

Transcriptome analyses of PBMCs by DNA microarray techniques are used in a wide variety and provide powerful tools to analyse biological responses to environmental challenge. For the bioinformatical conversion of these responses, that are usually revealed by large numbers of DE-genes, into well defined networks of molecular signaling the software Ingenuity Pathway Analyses is a proven reference. However, these techniques are known for some methodological issues to be taken into account which will be discussed in the following sections.

3.1.1 Advantages and Drawbacks of Microarray Analyses of PBMCs

Since the advent of microarray techniques (Schena *et al.* 1995) the simultaneous interrogation of large numbers of genes is possible. Before that time, information concerning expression levels of multiple genes were collectable only by extensive use of quantitative PCR techniques and later by so called macroarrays (Ledger *et al.* 2004).
The possibility of examining expression of up to tens of thousands of genes at once also causes a major issue called the multiple testing problem. It describes the increase of type I errors in statistical inference when multiple comparisons are performed within the same sample or data set. To circumvent this, several methods have been developed to recalculate levels of significance and allow multiple comparisons between two or more data sets: Holm-Bonferroni method (Holm 1979), Hochberg procedure (Hochberg 1988) and False Discovery Rate (FDR) by Benjamini and Hochberg (1995). In current microarray analyses the FDR is the most widespread and accepted method for correction in multiple comparisons (Storey and Tibshirani 2003). Concerning the presented publications related to this thesis the pilot study listed FDR-adapted p-values, so called q-values (Storey 2002), for each analysed time stage for information while for identification of DE-genes p-values were used. The succeeding main studies on divergent phenotypes applied an FDR algorithm provided by Ingenuity Pathway Analysis.

Typically, in transcriptomic microarray studies two conditions are compared, for example individuals, treatments or time-stages, that are assumed to be biologically different. Concerning the measured expression values which correlate directly to mRNA abundances fold change (FC) values are calculated which mean the factor by which abundances between the two conditions differ. In addition to pure p-values or FDR-corrected p-values, fold changes are often applied as threshold to filter for DE-genes. A review of microarray literature revealed that about half of published papers applied arbitrarily chosen fold changes of at least 2.0 (Laurent *et al.* 2013). Laurent and colleagues (2013) also demonstrated by highly sensitive RNA sequencing that biological changes in time course of inflammatory responses are based on small changes (FC < 2.0) of RNA quantities. Moreover, FC estimates derived from microarray data are commonly underestimated by factor two when compared to results based on quantitative PCR (Canales *et al.* 2006).
In the present thesis, only in the analyses of the first publication (Adler *et al.* 2013a, cf. chapter 2.2), whose significantly affected pathways often possessed both increased and decreased transcript abundances, a FC cutoff of 1.3 was applied in order to retain only DE-genes clearly different in transcript abundances. In contrast, the microarray data and enriched pathways

derived from divergent phenotypes (cf. sections 2.3 and 2.4) were characterized by uniformly increased or decreased transcripts with fold changes less than 2.0.
In summary, though high FC thresholds are often used in microarray analyses, they are rarely justified by biological considerations but often selected subjectively and may lead to loss of information. On the other hand, small changes can provide biologically relevant evidence (Bigler *et al.* 2013) but also increase the risk to include technical noise into the data.

cDNA microarray raw data contain expression measurements based on hybridization events to DNA oligonucleotides (probes or probesets) of which one or several correspond to a certain gene. The bioinformatical identification of a gene to the corresponding probeset is referred as to annotation and the quality of microarray raw data depends on the completeness and accuracy of the annotation process.
In general, microarray analyses can only cover genes whose derived oligonucleotides are present on the chip and which are correctly annotated. Genes not present on chip or not annotated will remain unrecognized. Otherwise, in rare cases gene annotation of single genes can be false what may lead to wrong identification of concerned genes.

In summary, for microarray data and succeeding interpretation of affected gene networks it should be taken into account that single results, i.e. single DE-genes, might be false positive or false negative due to the above-described issues of multiple testing and annotation. In addition, the application of FC thresholds may lead to unjustified exclusion of DE-genes with small but effective changes of mRNA abundances which might be crucial to recognise certain pathways. On the other hand, the usage of FC thresholds can be beneficial when resulting gene networks, i.e. canonical pathways, contain numerous DE-genes which exhibit ambiguous directions of fold change. In this case a FC threshold can help to evaluate the authenticity of the concerned pathway.

3.1.2 The feasibility of PBMCs to display systemic transcriptional responses

Analyses of PBMCs are widely used for immunological research and clinical diagnostics. Gene expression patterns in human PBMCs exhibits substantial inter-individual variation among healthy individuals (Radich *et al.* 2004) but this variation is considerably low when compared to alterations due to disease (Whitney *et al.* 2003). Transcriptional profiles of peripheral blood were shown to reflect numerous diseases (Liew *et al.* 2006) such as Arthritis (Barnes *et al.* 2004), Hypertension (Bull *et al.* 2004, Chon *et al.* 2004), Cancer (De Primo *et al.* 2003, De Vos

et al. 2002), Lupus erythematosus (Rus *et al.* 2002) as well as various environmental stressors (Wu *et al.* 2003) and sleep deprivation (Möller-Levet *et al.* 2013) .

High rates of correlation have been demonstrated between transcript abundances of PBMCs and other tissues or organs (Liew *et al.* 2006, Kohane *et al.* 2012). More than 80 % of gene expression were found to be shared between PBMCs and several other tissues including colon, stomach, liver, kidney, heart, brain and spleen. These findings and generally advantageous properties of blood sampling, such as sufficient availability by minimally invasive methods, led these authors to propose the usage of PBMC genomics as biomarkers for other tissues (Kohane *et al.* 2012) and for disease prognosis and diagnosis (Liew *et al.* 2006).

In addition to these advantages for systemic biomedical research, PBMCs are central in immunobiology-related transcriptome studies because they include the crucial cell types of the adaptive immune response. In human, microarray analyses of PBMCs enabled the discrimination between viral and gram-positive and -negative pathogen infection (Ramilo *et al.* 2006). In swine, one of the first technical feasibility studies on microarrays revealed a general inflammatory response to LPS and a TH1 predominated response to the mitogens PMA and ionomycin after *in vitro* stimulation of PBMCs (Gao *et al.* 2010). Likewise, a TH1 immune response was also found by Wilkinson and colleagues (2012) in PBMCs stimulated *ex vivo* with the mitogen Concanavalin A. Corresponding to one finding of this thesis (Adler *et al.* 2013a) IL-2 was found to be central in the porcine cellular immune response.

The approach to apply TT as experimental antigen in PBMC transcriptomics was proposed early after microarray techniques were established and a reliable pig genome assembly was available (Ponsuksili *et al.* 2008).

These approaches are complemented or contrasted by analyses of other immune-relevant tissues which are either permanently exposed to pathogenic microorganisms, e.g. the porcine lung (Zhao *et al.* 2006) or are key locations of adaptive immune response such as lymphoid tissues. As one example transcriptomic responses of pigs infected with *Salmonella enterica* were studied in vivo by microarray analyses on samples from mesenteric lymph nodes (Wang *et al.* 2007, Wang *et al.* 2008).

In the PBMC fraction leukocyte subtypes are present in different proportions with lymphocytes being the most frequent (70 - 90 %) followed by monocytes (~ 20 %) and dendritic cells (< 2 %) (see section 1.3). In turn, within lymphocytes T cells (CD3+) are the most frequent followed by B cells and NK cells. This uneven distribution can be expected to distort, to some extent, the detection of transcript abundance changes if these alterations are restricted to a certain cell type (McLaren *et al.* 2004). For example, if B cells were present in a frequency of 25% and T cells of 75% a duplication of B cell transcripts of a given gene would lead to an overall fold change of only 1,25 if T cell transcripts are not affected and remain constant.

This issue has to be kept in mind when interpreting PBMC transcript profiles, e.g. comparing T cell and B cell specific functions may result in underestimation of specific B cell function as illustrated by the example described above. Moreover, absence or low proportions of other immune cells in PBMCs such as monocytes, basophile granulocytes or dendritic cells probably prevents detection of their alterations. The alternative of microarray analysis of RNA isolated from whole blood is technically hampered by high contents of globin mRNA in whole blood that impair array sensitivity and signal variation and which require the application of globin mRNA reduction methods (Liu et al 2006).
In summary, while its uneven composition of leukocyte and lymphocyte subtypes should be taken into account, several favourable properties, i.e. involvement of T and B lymphocytes as crucial effectors of adaptive immunity, high proportion of shared gene expression to other organs and the easy availability of blood, make PBMCs a very suitable tool to access immune and other systemic transcriptome responses.

3.1.3 IPA as tool for bioinformative conclusions

Ingenuity Pathway Analysis is a widely used commercial software for analysing ‘omics data and modelling biological interactions based on the Ingenuity Knowledge Base (http://www.ingenuity.com). The key analytical tool is the assignment of DE-genes to canonical pathways.

While analysing the data presented in this thesis some properties and limitations of IPA turned out to have to be taken into account in the evaluation of bioinformatical analyses. IPA’s canonical pathways contain two major groups: metabolic and signaling pathways. The signaling pathways in turn contain categories such as cellular immune response, humoral immune response, cytokine signaling, pathogen-influenced signaling, cellular growth, proliferation and development, growth factor signaling and organismal growth and development. Often a given pathway is found in two or more categories what makes it difficult to consider and compare the representation of these categories when extensive data should be assessed in a summarizing manner. For example the “Antigen Presentation Pathway” is found in both the ‘humoral’ and ‘cellular immune response’ category, the pathway “Role of NFAT in Regulation of the Immune Response” is grouped in ‘cellular immune response’, ‘humoral immune response’ and in ‘intracellular and second messenger signaling’. Furthermore, as the differentiation between cellular and humoral immune response is one major topic in this thesis, it has to be taken into account that the IPA category for humoral

immune response provides only 17, whereas the category cellular immune response lists 70 different canonical pathways. Since several pathways of the cellular immune response group share a lot of genes a given list of DE-genes will often result in an enrichment of those respective pathways. As example the pathways “T Cell receptor signaling”, “CD28 signaling in T helper cells”, “PKCθ Signaling in T Lymphocytes” and “Role of NFAT in Regulation of the Immune Response” all include Phosphatidylinositol 4,5-bisphosphate, T cell receptor and ZAP70, CD3, CD4, CD28, BCL10, FOS, FYN and others more. This may lead to an over-representation of cellular immune response pathways beside humoral immune and other responses in a result list of most significant canonical pathways upon analysis of a set of DE-genes containing those genes. However, humoral immune responses and other cellular responses may be composed of a similar number of DE-genes but due to lower overlap of related pathways they will appear less represented.

Furthermore, IPA analyses cannot be configured for any tissue-specific aspects of biological interaction that would allow the exclusion of artefact findings such as “Alzheimer’s disease signaling” in liver tissue of juvenile animals. Hence, the identified pathways must be evaluated by the investigator.
Canonical pathways (and other predicted molecular networks) are rated after calculation of an overall p-value (by an unpublished algorithm) by taking into account the single p-values of DE-genes and the numerical ratio between these genes and all genes constituting each pathway. However, the consistency of routes of expression changes (up- or down-regulation) are not considered. Thus, a canonical pathway might be found significantly affected that, for example, contains five up-regulated genes and five down-regulated genes which interact with each other. If the up-regulated genes were activators and the down-regulated were inhibitors one would conclude that this pathway is highly activated. But if all genes were activators one would conclude that up- and down regulation will cancel out each other. Again, the identified pathways must be verified carefully for plausibility of the underlying changes of transcript abundances.

3.2 The PBMC response to TT vaccination

The porcine PBMC transcriptome is subject to extensive significant and functional transcript abundance changes throughout the immune response due to TT vaccination. Affected genes were related to several functions such as immune response, cellular and organismal growth, proliferation, development and cell-cell communication. A pilot time course study of tight intervals revealed notable early transcriptional alterations by cytokine and T cell associated responses, the following chapter discusses the authenticity of these findings and the implications for ongoing work.

In these main studies, in order to dissect the responsiveness of varying phenotypes with divergent backgrounds of high and low lean growth and AB titers, respectively, two different approaches were performed. First, the statistical comparison between all four differentiated phenotypes obtained by combination of high and low lean growth and AB titers, respectively. Second, the statistical comparison between time intervals performed separately for each differentiated phenotype. Subsequently, these obtained time-course profiles were compared among the different phenotypes. The first approach which weighted time stages equally was performed to indicate the general significance of LG phenotypes since early in data monitoring it became obvious that divergent LG phenotypes show clearly different transcriptional patterns. The second approach was set-up to analyse the impact of the time component, in particular the effect of an adaptive immune response as observed in the transition from day 14 to day 28.

3.2.1 Responses observed by time course study of pooled subsets

A pilot study has been conceived in order to gain first insights into PBMC transcriptional response to *in vivo* immune stimulation (Adler *et al.* 2013a). A subset of 18 animals equally divided into three groups has been vaccinated and microarray analyses have been performed for 2, 4, 8, 30 and 75 hours after the initial and the booster vaccination, respectively. Throughout these tight time intervals high numbers, i.e. from several hundreds to more than 1100, of differentially expressed genes were identified. Related functions were attributed predominantly to immune responses and to numerous further biofunctions.

Significant changes of mRNA abundances were observed remarkable early, i.e. at 2 h, 4 h and 8 h after the initial vaccination. During these early time points cellular immune response and

cytokine signaling were among the predominant significant pathways. A similar study reports transcriptional immune response covering TH1 and TH2 markers in spleen cells isolated from TT vaccinated mice already 4 h after *in vitro* TT-restimulation (Regnström *et al.* 2002). In contrast, time course analysis of ConA stimulated porcine PBMC culture revealed at 3 h only 46 DE-genes while at 20 h and at 68 h ten times more genes were affected (Wilkinson *et al.* 2012).

Our observation of early cytokine signaling events are in accordance with in vitro studies of transcriptional responses to immune stimulation that have shown certain cytokine mRNA to be expressed within 30 minutes whereas others were detectable only later (Zhong *et al.* 1993). More unsuspected, as activation of innate immune responses may be expected, was the observation of T cell related immune activation already two hours after antigenic challenge because many of these signaling processes depend upon antigen presentation to T cells that can be expected to be a time consuming event.

The overall process of antigen presentation comprises antigen uptake and protein degradation, MHC synthesis, trafficking of peptide-MHC complexes to the cell surface and display (Kirschner *et al.* 2007). Antigenic proteins in the cytoplasm of professional antigen presenting cells (i.e. macrophages, dendritic cells or B cells) are degraded enzymatically to short peptides. In the case of extracellular antigens, the proteins first become internalized into the cytoplasm by endocytosis before processing to peptides. Following MHC synthesis binding of these peptides to either MHCI or MHCII generates peptide-MHC complexes that are transported to the cell surface. The final step is the display (presentation) of these complexes to T cell receptors of either CD4-positive Helper T cells (in case of MHCII) or CD8-positive cytotoxic T cells (in case of MHCI). Kirschner *et al.* (2007) provide estimates of time scales on which antigen presentation may occur. At the cellular level it takes several seconds to minutes while at tissue level scales of 10^4 to10^5 seconds are considered.

It has to be taken into consideration that antigen presentation generally takes place at two distinct sites of the host organism: at the infection site both macrophages and dendritic cells take up antigens, but it are the dendritic cells that migrate to the lymph nodes where they present antigens to naïve t cells while macrophages stay at the infection site. In addition, these two types differ in efficiency and rate of antigen presentation as dendritic cells are much more efficient than macrophages (Kirschner *et al.* 2007).

Concerning our findings of cytokine and T-cell signaling early in time course it remains unclear if these alterations are due to antigen presentation by dendritic cells in lymph nodes or by macrophages in the periphery. However, because it is well known that naïve CD4 stimulation must occur in lymph nodes (Itano and Jenkins 2003) fully functional TH1 and TH2 signaling can be considered as authentic only for later time intervals.

Throughout the following time intervals (>8 h) cellular immune response together with cellular and organismal growth, proliferation and development and second messenger processes were predominantly affected. However, analyses of these responses is to some extent hampered by the fact that several significant pathways were not consistently composed of unidirectionally altered gene products but rather contained both increased and decreased transcript abundances within the same signaling pathway. Such restriction was not seen for the expression profiles obtained by the follow-up studies of different phenotypes whose significant pathways generally contained either only increased or only minored transcript abundances.
In conclusion, the transcriptional responses observed by the pilot study showed a broad response involving cellular and cytokine driven immune responses whereas humoral immune responses were found underrepresented. Though these immune responses became significant very early among the later time intervals no outstanding pathway or a significant gene network was found that could give reasons for focussing to a particular time interval after immunization. For ongoing analyses the time interval of 14 days after each vaccination was chosen for which by measurements of anti-TT titers a phenotypic equivalent was available. Microarray analyses were thus performed at time intervals when information at protein level can be combined with transcriptomic data.

3.2.2 Divergent phenotypes show distinct responses

Based on the pilot study and the growing interest in understanding the biological interrelation between immune traits that are beneficial for disease resistance and traits of production performance the centre of our interest was to elucidate differences of transcriptomes for differing phenotypes for these traits. Therefore, a study was designed to record transcriptomic responses of PBMCs to experimental TT vaccination against the backgrounds of divergent lean growth and divergent anti-TT antibody titers and to compare the resulting response profiles among phenotypes.
There are numerous reports comparing the transcriptomes of pig breeds that differ in lean-to-fat ratio, but these studies compare genetics of different almost closed populations, i.e. breeds, without consideration of any interrelation to other traits. To authors knowledge the present studies are the first reports on transcriptomic comparisons of combined phenotypes differentiated for divergent leaness and divergent immune responsiveness.

A general comparison between high LG and low LG over all three time stages (weighted equally) without differentiation of AB phenotypes revealed higher transcript abundances in high

LG animals for the most significant canonical pathways and predicted biofunctions (cf. section 2.2, Adler *et al.* 2013b). These gene transcripts were related to T Cell activation by antigen-presenting cells, such as T-cell receptor, PKCθ, and CD28 signaling and increased development, differentiation, and homeostasis of lymphocytes. The more differentiated comparison of divergent LG phenotypes again demonstrated those findings, i.e. higher mRNA abundances of T Cell mediated responses for high LG compared to low LG with the background of hiAB phenotypes. No significant findings were detectable for the same comparison but against the background of loAB. Therefore, only animals producing high AB titers show also a clear difference between divergent LG phenotypes, i.e. higher mRNA abundances for T cell-related immune response.

In contrast, comparison of divergent AB phenotypes did not reveal immune functions among the most significant affected signaling pathways. Interestingly, low AB phenotypes possessed generally higher transcript abundances than high AB phenotypes related to functional gene networks that differed clearly between high LG or low LG backgrounds. Low AB phenotypes had higher activation of cell-mediated immune response and lymphocyte differentiation or activation when compared to high AB with the background of low LG (loLG+hiAB vs. loLG+loAB). One might suppose that the higher response on T cell level reflects a compensation of lower humoral response ability.
Against the background of high LG (hiLG+hiAB vs. hiLG+loAB) the low AB phenotype had higher activation of cellular and organismal growth, proliferation and development as well as intracellular and second messenger signaling pathways. Predicted biofunctions were related to activation of cell transformation, aggregation, binding and survival. Hence, this comparison of divergent AB phenotypes did not indicate any compensatory effects as were presumed for the counterpart of divergent AB under low LG background.
In conclusion, the differentiated comparisons of divergent AB phenotypes indicate that divergent AB titer phenotypes, which were characterized by ELISA quantification of anti-TT antibodies in plasma, are not reflected by mRNA profiles that can explain any AB related humoral immune response. Compensatory effects between humoral and cellular immune responses may be presented by the finding that low AB phenotypes clearly showed higher transcript abundances for cellular immune responses. However such reverse effects became visible only in the subgroup of low LG phenotypes.

3.2.3 Phenotype-related differences appear in response to the booster vaccination

It is reasonable that the initial TT vaccination induces an innate immune reaction since weaning piglets can be regarded as unprimed for the unubiquitous TT antigen, whereas the second vaccination after two weeks induces an adaptive immune response. The interesting question arises at which stages the differences between phenotypes emerged. The transcriptional profiles that revealed differences between phenotypes were dissected in order to differentiate between the initial response to the first vaccination and the secondary response to the booster vaccination (cf. section 2.3, Adler *et al.* 2015).

Both subgroups of high LG clearly revealed the increase of transcripts after the booster vaccination and provide convincing indications for the enrichment of canonical immune pathways. These pathways were related to antigen presentation, T Cell and B Cell activation and signaling, but the most indicative pathway for humoral immune response, B Cell receptor signaling, was observed only in hiLG+hiAB to be among the most significantly activated immune function. In contrast, for the hiLG+loAB phenotype, the canonical pathway B cell development was most significant what may indicate a delayed response of loAB compared to hiAB concerning B cell proliferation or AB production. The other significant pathways were shared between both hiLG groups and are related to the enrichment of pathways of T cell activation by antigen presentation and costimulation.
Therefore, at transcriptomic level, hiLG phenotypes present a pronounced cellular immune response that was combined with clear activation of humoral immune response only in animals that exhibit actually high AB titers in plasma (Fig. 3). This demonstrates conformity between transcriptome information and actual phenotypes that had been characterized at the protein level.
Both subgroups of low LG revealed comparatively weak responses to the booster vaccination (Fig. 3). For the background of high AB only few significant pathways and DE-genes associated with immune responses were found. loLG+loAB presented numerous significant pathways but only few of them were composed by uniformly upregulated or downregulated mRNA abundances.

As was observed in the pilot study (cf. section 2.1, Adler *et al.* 2013a) and in the comparison of phenotype-specific primary responses (cf. section 2.3, Adler *et al.* 2015) the transition between day 0 to day 14 revealed a general decrease of transcript abundances. Although several growth factor pathways were represented in the hiLG+hiAB group and numerous immune pathways in both loAB phenotypes a clear pattern of phenotype-related functions could not be inferred. Since for four time intervals during 24 hours following the initial

vaccination transcripts increased the observation of an overall decrease at day 14 might be explained by reorganization events in tissues or leukocyte composition. Also ontogenetic processes should be taken into account that possibly influence the dynamics of mRNA abundance changes.

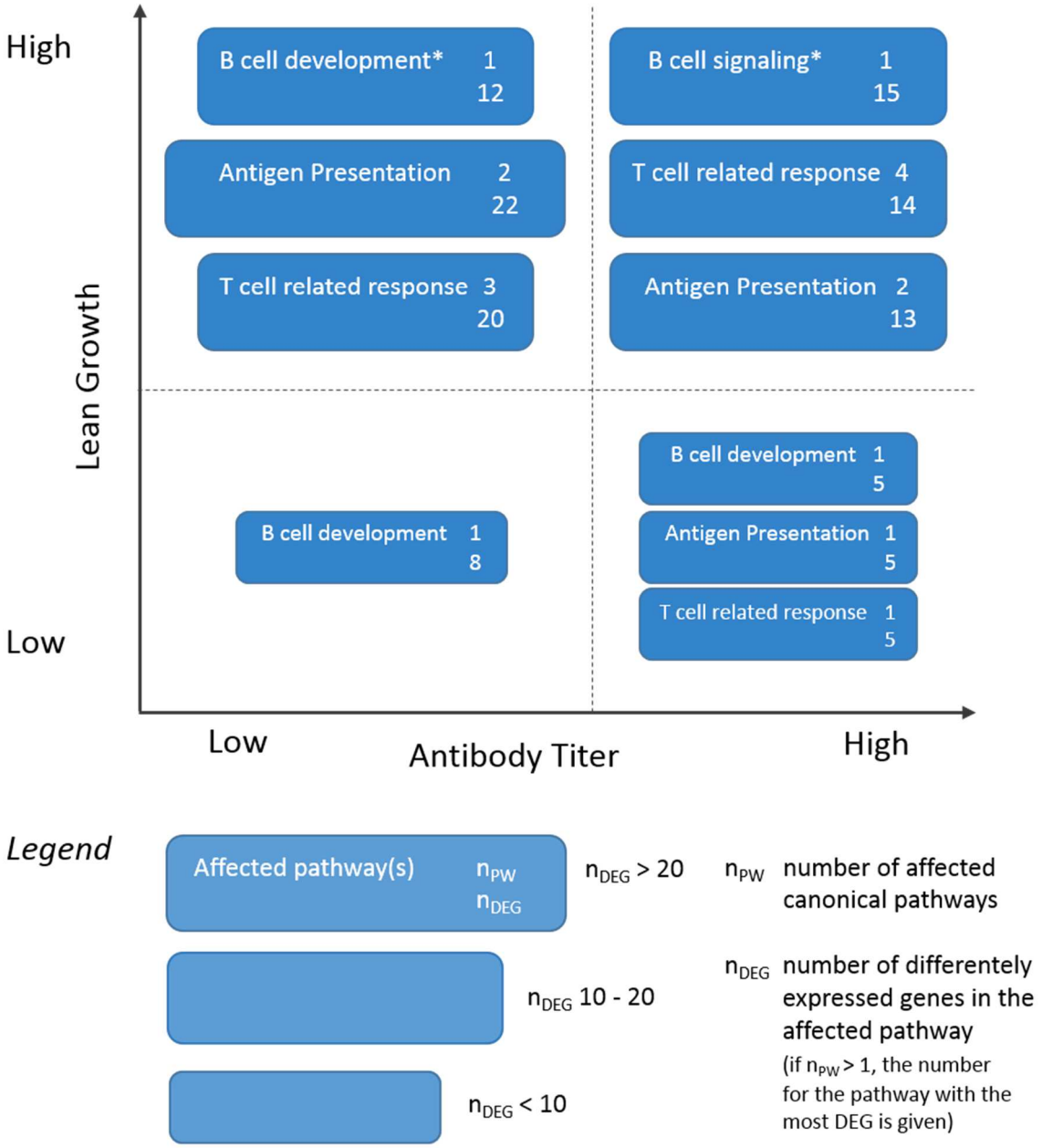

Figure 3. Interpretation of phenotype-specific activation of signaling pathways during adaptive immune responses to the booster vaccination. For each phenotypic group of high and low LG or AB titers, respectively, the three most significant representatives of canonical pathways are given in a blue box. The box size is drawn in relation to the numbers of DE-genes leading to the respective pathway in order to illustrate the relative strength of each phenotype's response. In the boxes, on the right the upper number (n_{PW}) indicates the sum of associated significant pathways, the lower number (n_{DEG}) counts the number of DE-genes in the pathway(s). The asterisk indicates the most significant pathway in the respective result subset.

3.3 Implications for Leaness and Immunocompetence

The results of the presented transcriptome profiling studies as well as numerous literature reports point out that lean body composition and immune response are not mutually exclusive and can be reconciled. Anti-tetanus AB titers as determined here can not be considered as an appropriate marker to reflect transcriptomic immune responses. However, the correlation of high lean growth with enhanced immune gene transcripts suggests leaness to be linked to at least some traits of immunocompetence. However, by our findings and observations by other authors such as that under restricted but not under *ad libitum* feeding high lean growth animals had higher proliferation of specific lymphocytes and monocytes (Clapperton *et al.* 2006) the interplay between leaness and immunity turns out to be complex and an overall-picture is anything but clear-cut. It remains to be further examined if genetic predisposition to lean growth also drives immunocompetence or, if the other way round immune genes are linked to physiological performance.

A possible explanation can be provided by consideration of the GH-IGF-1 axis. Pig selection for lean body composition and growth rate has been shown to lead to changes of the GH-IGF-I axis including higher expression of GH (te Pas *et al.* 2001, te Pas *et al.* 2004). The pituitary growth hormone (GH) and its mediator Insulin-like growth factor 1 (IGF-1) are primarily known as key hormones for growth and development. In addition, they markedly modulate development and function of the immune system (van Buul-Offers *et al.* 1998, Heemskerk *et al.* 1999). Numerous immune cells such as mature spleenic B and T cells express GH and type I IGF receptors. Rodent models revealed that GH is important for splenic growth (van Buul-Offers *et al.* 1998). The effects of IGF-1 on proliferation, function and maturation of immune cells can be summarized as follows: T and B cells show a high affinity binding of IGF-1 (Stuart *et al.* 1991) and expression of IGF-1R on T-cells is inducible by mitogenic stimulation (Xu *et al.* 1995). IGF-1 was shown to be crucial for thymus and spleen size due to increased numbers of spleen and thymus resident lymphocytes (Rosenfeld *et al.* 1994, Murphy *et al.* 1995) IGF-1 pretreatment causes an enhanced primary antibody response when compared to non-treated controls (Robbins *et al.* 1994). Moreover, IGF-1 has a prominent effect in lymphocyte maturation as it regulates lymphoid hematopoiesis even before migration to secondary lymphoid organs (Geffner 1997) and it enhances the differentiation of thymic T-cell progenitors (Gjerset *et al.* 1990) and is also involved in the differentiation of bone marrow pro B-lymphocytes (Funk *et al.* 1994). Beside the direct effects of GH - IGF1 axis on immune function and cell proliferation the evident role in lymphocyte precursor maturation suggests that animals

with higher activity of the GH - IGF1 axis may have an advantage of immune status in terms of early acquired immunocompetence or host-defence.
te Pas and colleagues (2004) proposed a model that explains selection for lean body composition to be correlated with increased GH mRNA, number of GH pulses and increased hepar IGF-1 synthesis. In light of the above described numerous effects of GH and IGF-1 on lymphocytes it may be reasonable that the observed predominant immune responses of high lean growth animals are due a higher activation of the GH-IGF-1 axis.

Another point of view on the interplay between leaness and immune system can be inferred from recent obesity research. In obese human and mouse models there is a strong pathophysiological relationship between inflammation in adipose tissue, muscle and liver and insulin resistance (Olefsky and Glass 2010). In obesity, macrophages accumulate in adipose tissue and the numbers of macrophages correlate to the degree of obesity with ~40% macrophage cell content in obese compared to ~10 % in lean individuals (Weisberg *et al.* 2006).
The secretion of proinflammatory cytokines and chemokines by macrophages leads to the alteration of insulin target cells. Paracrine effects of TNFa, IL-6, IL-1ß and others activate several serine kinases, including JNK (c-Jun N-terminal kinase) and IKK (Inhibitor of κB kinase), that in turn activate transcription factor targets such as c-Jun/Fos and NF-kB. These factors then stimulate transcription of multiple inflammatory pathway genes (Hacker *et al.* 2006, Nguyen *et al.* 2007). Moreover, those serine kinases also phosphorylate insulin receptor substrate proteins, insulin receptors and probably other insulin signaling components and thus impair the normal insulin signaling in insulin target cells and finally lead to the state of insulin resistance (Aguirre *et al.* 2000, Hirosumi *et al.* 2002).
Although macrophages themselves can recruit more macrophages into adipose tissue in a positive feedback manner, activated T cells, in particular TH1 lymphocytes, have been shown to be major effectors of macrophage infiltration into adipose tissue and the resulting inflammation state (Kintscher *et al.* 2008, Winer *et al.* 2009). At least in view of this participation of the adaptive immune system the question raises of whether in obese humans or animals the general capability of the immune system could be affected in case of antigen challenge by pathogen infection or vaccination (Bandaru *et al.* 2013). In human strong correlation has been shown between obesity and increased risk, amongst others, for nosocomial (Choban *et al.* 1995, Kaye *et al.* 2011), respiratory (Jedrychowski *et al.* 1998) and urinary tract infection (Semins *et al.* 2012). Furthermore, clear association to increased mortality in influenza (Louie *et al.* 2011, Van Kerkhove *et al.* 2011) and side effects of hepatitis C (Lo Iacono *et al.* 2007) have been shown. Although not in all cases the inflammation process in adipose tissue as described above must be the sole cause leading to increased susceptibility for infection these

studies present convincing evidence for a negative correlation between obesity and immunocompetence in terms of host defense.

In addition, impaired immunocompetence of overweight or obese individuals has also become visible in response to vaccination (Sheridan *et al.* 2012, Bandaru *et al.* 2013). One of the earliest studies has found high BMI to be associated with undetectable AB titers against Hepatitis B immunization (Weber *et al.* 1985). Lower antibody response was also found to be correlated in overweight adolescents to tetanus vaccination (Eliakim *et al.* 2006).

However, the mechanisms leading from the inflammation state in adipose tissue or muscle to an impaired systemic immune competence are still poorly understood (Bandaru *et al.* 2013). Paracrine adipokine and cytokine release by macrophages or lymphocytes in adipose tissue can also have endocrine effects (Olefsky and Glass 2010), thus, effecting proinflammatory stimuli distributed to other tissues which have to be assumed to cause adverse effects for systemic immune balance and impaired immunocompetence in case of pathogen encounter (Bandaru *et al.* 2013). Sheridan and colleagues (2009) have shown in mice that antigen presentation by dendritic cells is impaired in obese animals. In addition, impaired functionality and lowered activation states of CD4+ and CD8+ T lymphocytes has been demonstrated for overweight and obese humans in response to H1N1 influenza (Paich *et al.* 2013).

Interestingly, in contrast to expectations, the phenotypic read-out of anti-TT titers was not found to be associated with a general enrichment of immunological pathways. Only a part of differentiated comparisons could explain significantly affected pathways by divergent AB titers. These include on the one hand the responses of high LG animals to booster vaccination while high AB animals also possessed B Cell Receptor Signaling as the mostly activated pathway and low AB did not show such activation of a humoral response. On the other hand, some indications have been obtained that low AB phenotypes respond with a comparatively high cellular immunity possibly compensating limited ability to establish humoral responses.

Therefore, the phenotype of high lean growth rather than high AB titers turned out to be closely associated with high transcriptomic immune responses during the adaptive immune response. Conclusions on the suitability of AB titers as phenotypic markers for immunocompetence should be drawn cautiously since both transcriptomic profiles and AB titer assays represent only snapshots of tight time intervals possibly leaving biological dynamics unrecognized.

3.4 Scientific relevance and future perspectives

The presented transcriptome studies provide clues to the relationship of phenotype expression between lean growth and immune response to vaccination in pig. The central observation is that the phenotype of high lean growth was found to be associated with increased transcript abundances of antigen presentation, and T cell and B cell related immune responses. To confirm and refine these results ongoing research under several aspects is required.

The presumption of high cellular immune response as reflected by transcript profiles should be confirmed at the protein level by measuring actual parameters of cellular immunity. Furthermore, since PBMCs are heterogenous and unevenly composed the quantification of leukocyte subtypes by flow cytometric techniques (Pasternak *et al.* 2014) can help to explore whether the frequencies of leukocyte subtypes change in course of immune immune response. This could enable a correction for proliferative events, as for instance assumed by Wilkinson *et al.* (2012), which mask small changes in immune biofunctions. If frequencies of certain T or B lymphocytes are not significantly changed in response to immune stimulation the observed shifts of transcript quantities can be expected to be due to gene regulation events. Vice versa, if considerable changes of PBMC composition could be observed proliferative events can be assumed as effectors of immune response. Depending on which process will turn out to be crucial, different key proteins of gene regulation or proliferation and their genetic variation will become of interest to explain the variability of phenotype expression.

If independent analyses of biological replicates confirm the association between high lean growth and enhanced immune responses the possible influence of haplotypes or genotypes can be examined - for example by the use of genome wide single nucleotide polymorphism (SNP) genotyping.

Eventually, it remains to be investigated to what extent high immune responses to experimental stimulation in the present context are reflected by actual disease resistance in pig farming. The final goal is a balanced breeding for health and welfare including an extensive disease resistance in combination with consistent economic production. To progress in this, a detailed and comprehensive understanding of the involved genetic effectors shall be aspired.

Summary

Livestock species undergo artificial selection for desired phenotypic changes including increased weight gain, lean growth or milk production. For the domestic pig *Sus scrofa domestica* positive and negative association between immune and performance traits as well as positive correlation between performance and certain immune traits but simultaneously adverse effects to other immune traits have been observed. Little is known about the balance of underlying genetic factors, however, modern pig breeding requires the inclusion of the immune response in order to address disease resistance, animal health and welfare. Moreover, information about the interrelation between porcine immune system and physiology can be helpful for human medicine since the pig is more and more recognized as suitable model for functional genomic research.

Here, studies on porcine immune responsiveness are based on the application of tetanus toxoid (TT) vaccine as experimental antigen to induce a comprehensive cellular and humoral immune response. Subsequently, the response to TT was studied by microarray analysis of peripheral blood mononuclear cells (PBMC) transcriptome profiles. Differences of relative mRNA abundances between time stages and phenotypes led to the identification of differentially expressed genes which could be assigned to affected molecular signaling pathways. Analyses of these responses of divergent phenotypes differentiated for high and low lean growth performance and high and low anti-TT (AB) titers, respectively, did not result in the identification of immune response pathways if high AB were compared to low AB phenotypes. However, the results clearly identified high lean growth to be associated with increased transcript abundances for multiple signaling pathways of the adaptive immune system. Furthermore, a differentiation of responses to the initial and to the booster vaccination, respectively, revealed that phenotype-related differences appear not until the response to the booster vaccination. In this time interval high lean growth animals activate numerous immune response pathways including B cell signaling as most significant in the subgroup of high AB but T cell signaling as predominant in low AB animals.

Explanatory approaches for the interrelation between lean growth and immune responsiveness can be derived from literature reports that, on the one hand, show that pig selection for lean body composition influences the GH (growth hormone) - IGF-I (insulin-like growth factor 1) axis which in case of high activation can be expected to provide an advantageous immune status. On the other hand, low lean growth, i.e. fat accumulation can be seen as to be a precursor of obesity and recent research on obese human and mice has shown chronic inflammation and insulin resistance in adipose tissue, muscle and liver that eventually cause an impaired systemic immunocompetence.

Zusammenfassung

Nutztiere erfahren durch künstliche Selektion gewünschte phänotypische Veränderungen, z.B. schnellere Gewichtszunahme, erhöhter Magerfleischanteil oder gesteigerte Milchproduktion. Für das Hausschwein *Sus scrofa domestica* konnten sowohl positive als auch negative Zusammenhänge zwischen Immun- und Leistungsmerkmalen, sowie positive Korrelationen zwischen Leistung und bestimmten Immunmerkmalen einerseits, aber gleichzeitiger Benachteiligung weiterer Immunmerkmale andererseits, beobachtet werden. Über die genetischen Faktoren dieser Wechselwirkungen in der Merkmalsausprägung ist wenig bekannt, jedoch kommt Merkmalen der Immunantwort eine hohe Bedeutung innerhalb moderner Zuchtprogramme zu, um Ansprüche hinsichtlich der Krankheitsresistenz, der allgemeinen Tiergesundheit und des Wohlbefindens der Tiere zu gewährleisten. Darüber hinaus kommt dem Zusammenhang zwischen Immunsystem und Physiologie auch für die moderne Biomedizin eine hohe Bedeutung zu, da das Schwein zunehmend als Modellorganismus, u.a. für die funktionelle Genomanalyse, erkannt wird.

Die vorliegenden Untersuchungen der Immunkompetenz basieren auf der Anwendung von Tetanustoxoid (TT) als experimentelles Antigen, um eine umfassende, zelluläre und humorale, Immunantwort auszulösen. Im Anschluss wurde die Reaktion auf TT mit Hilfe von Microarray-Analysen der Transkriptomprofile von peripheren einkernigen Leukozyten (PBMC) untersucht. Unterschiede der relativen mRNA-Häufigkeiten zwischen verschiedenen Zeitstadien und Phänotypen ermöglichten die Identifizierung von differentiell exprimierten Genen, die wiederum veränderten molekularen Signalwegen zugeordnet werden konnten. Diese Untersuchungen von divergenten Phänotypen bezüglich eines hohen und niedrigen Magerfleischanteils sowie hoher und niedriger TT-Antikörpertiter konnten für den Vergleich letzterer keine signifikanten Signalwege der Immunantwort aufzeigen. Jedoch ergaben die Ergebnisse eindeutige Hinweise, dass das Merkmal Magerfleischanteil mit erhöhten Transkriptabundanzen für zahlreiche Signalwege der adaptiven Immunantwort assoziiert ist. Weiterhin zeigten differenzierte Vergleiche zwischen Reaktionen nach der ersten und der zweiten Impfung, dass die phänotyp-spezifischen Unterschiede erst mit der zweiten Impfung hervortreten und dass dabei Tiere mit hohem Magerfleischanteil zahlreiche Immunsignalwege aktivieren, innerhalb derer für die Merkmalskombination hoher Magerfleischanteil und hohe Antikörpertiter der B-Zell Rezeptor Signalweg und für einen hohen Magerfleischanteil kombiniert mit niedrigen Antikörpertitern verschiedene T-Zell Signalwege vorherrschend vertreten sind.

Mögliche Erklärungsansätze für den beobachteten Zusammenhang zwischen Magerfleischanteil und Immunkompetenz können aus Erkenntnissen anderer Autoren bezüglich der GH (growth hormone)-IGF-I (insulin-like growth factor 1)-Achse und aus dem

gegenwärtigen Wissen über Adipositas abgeleitet werden. Zum einen konnte gezeigt werden, dass die Selektion auf einen hohen Magerfleischanteil beim Schwein eine höhere Aktivität der GH-IGF1-Achse bewirkt, was im Anschluss zu einem vorteilhaften Immunstatus führen kann. Zum anderen ist bekannt, dass Übergewicht als Gegensatz zu Magerkeit, v.a. in der gesteigerten Form der Fettleibigkeit oder Adipositas, beim Menschen und im Mausmodell, einen Zustand der chronischen Entzündung und Insulinresistenz in Fettgewebe, Muskel und Leber bewirkt, der schließlich die systemische Immunantwort nachhaltig beeinträchtigt.

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List of abbreviations

AB	Antibody, antibodies
DE-genes	Differentially expressed genes
ELISA	Enzyme linked immunosorbent assay
IPA	Ingenuity Pathway Analysis
LG	Lean growth
MHC	Major histocompatibility complex
PBMC(s)	Peripheral blood mononuclear cell(s)
SLA	Swine leucocyte antigens
TH1 (2)	T helper cells, type 1 (2)
TT	Tetanus toxoid

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01/2005 - 09/2006	Internchip and external diploma thesis at the University of Göttingen, Historical Anthropology: ancient DNA analysis

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Publications

Journal articles

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Marcel Adler, Yang Du and Klaus Wimmers: GeneDialog: Funktionelle und epistatische Netzwerke von Genen metabolischer und immunologischer Funktionswege sowie QTL für Immun- und Produktionsmerkmale. FUGATO-Statusseminar, 09.-10. Februar 2011, Kassel

Marcel Adler and Klaus Wimmers: FUGATOplus-Projekt GeneDialog: Funktionelle Netzwerke von Genen metabolischer und immunologischer Funktionswege nach Vakzination mit Tetanustoxoid als Th1/Th2 Modellantigen. FUGATO-Statusseminar, 14.-15. Oktober 2009, Kassel

Rabea Kleindorp, Marcel Adler, Maximilian Westenthanner, Bernd Herrmann, Susanne Hummel: aDNA preservation patterns within different human skeletal elements and in dependance on different chromosomal locations. 8th International Conference on Ancient DNA & Associated Biomolecules: 16-20 October 2006, Lodz.

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Erklärungen

Die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena vom 4.12.2012 ist mir bekannt. Die vorliegende Dissertation habe ich selbstständig verfasst und keine anderen Quellen und Hilfsmittel als die von mir angegebenen verwendet. Es wurden keine Textabschnitte eines Dritten oder anderer eigener Prüfungsarbeiten übernommen. Unterstützt bei Auswahl und Auswertung des Materials und bei der Herstellung des Manuskripts wurde ich durch Professor Dr. Klaus Wimmers.
Die Hilfe eines Promotionsberaters habe ich nicht in Anspruch genommen. Es haben keinerlei Dritte, weder unmittelbar noch mittelbar, geldwerte Leistungen von mir im Zusammenhang mit der vorliegenden Dissertation oder deren Inhalt erhalten.

Ich habe die Dissertation bei keiner anderen staatlichen oder wissenschaftlichen Institution als Prüfungsarbeit eingereicht. Weiterhin habe ich weder die vorliegende noch eine in Teilen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation eingereicht.

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